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# FOOD RESERVES AND THEIR TRANSLOCATION TO THE CROWN BUDS AS RELATED TO COLD AND DROUGHT RESISTANCE IN ALFALFA[1]

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## INTRODUCTION

The physiological role of food reserves in many crop plants has been studied and reported to be a factor in the hardening process of plants to different adverse conditions. Time-of-cutting experiments with alfalfa (*Medicago sativa* L.), conducted by the Division of Forage Crops and Diseases in cooperation with the Kansas Agricultural Experiment Station, at Manhattan, Kans., during the last 35 years, have demonstrated the effects of different cutting treatments on hay quality, on longevity of stands, and on the storage of carbohydrates and nitrogen in the roots.

The study reported herein was made to determine the importance of food reserves in relation to the physiological processes that occur during the hardening of alfalfa to adverse conditions. This study was designed particularly to show the effect of time of fall cutting on the development of the crown buds and the effect of stored organic food reserves in the roots on their cold resistance. As used in this paper, the term "crown buds" is defined as the new growth, either pink or white in color, originating from the crown or crown shoots under the surface of the soil. As soon as a bud developed a green tip it was no longer classified as a crown bud.

## REVIEW OF LITERATURE

Nelson (*17*)[3] reported that a reduction of vigor and productivity accompanied a lowering of food reserves in alfalfa roots, and other investigators (*5*, *11*) have obtained similar results. The present writer (*7*) found that the critical period in the life of alfalfa is in the fall and that removal of the late summer growth early in the fall lowered the carbohydrate and nitrogen content of the roots to such an extent as to make the plants more susceptible to winter injury. Rather and Dorrance (*21*) reported that grazing in the fall reduced the dry matter in the roots and the number and vigor of crown buds. Electrical conductivity tests of the roots indicated that alfalfa ungrazed after August 28 hardened off much better.

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[3] Italic numbers in parentheses refer to Literature Cited, p. 45.

Silkett et al. (*24*) concluded that alfalfa plants cut in September develop fewer crown buds per plant than those cut earlier or during October. Johnsson (*12*) found that higher yields were associated partly with a larger amount of reserve food stored in the roots and partly with a greater number of winter-hardy crown buds.

Ireland (*10*) noted a difference in fluctuations of the cell-sap concentration during the winter in hardy and nonhardy varieties of alfalfa. Dexter (*2*) stated that "hardening of plants is favored by conditions which tend toward the accumulation or conservation of carbohydrates and other reserve foods." Kneen and Blish (*13*) showed a positive correlation between cold resistance and sucrose content in winter wheat plants. The writer (*7*) found that conditions of top growth favorable for high food storage in alfalfa can be brought about by proper cutting practices.

The work of other investigators has established that, coincidental with the increase in drought resistance in some plants, there is a pronounced increase in some carbohydrates and a decrease in others. Lvoff and Fichtenholz (*14*) and Vasiliev and Vasiliev (*26*) noted that following wilting there was an increase in the hydrolysis of starch and an increase in sugars.

These experiments, as well as others, suggest that the hardening processes to cold and to drought are similar and may be controlled to some extent in alfalfa by proper cutting practices.

Dunn (*4*) demonstrated that the protoplasmic constituents of the cells of alfalfa are of primary importance in the hardening to cold. Newton and Martin (*20*) found that the amino nitrogen content of the wheat plant increased during the hardening process and that high carbohydrate and nitrogen content are necessary to cold resistance. Harvey (*9*) held that the splitting of protein into less easily precipitated forms is a method of adaptation of plants to cold. Mark (*15*), working with alfalfa, found a marked increase in the content of both protein and nonprotein nitrogen from uncut plants while that of continuously cut plants remained about constant, indicating that, if a high content of protein nitrogen is related to cold resistance, there is a distinct difference in favor of uncut plants. Greathouse and Stuart (*8*) concluded that a high concentration of total sugars and total nitrogen was associated with cold resistance. Tysdal (*25*) reported that the concentration of sugar and amino acids, as found in the extract of alfalfa roots, influenced the protective activity. Miller (*16*), in a discussion of synthesis of proteins by green plants, stated that proteins are not formed in the plant without a supply of carbohydrates. Thus there probably is a close association of the stored food reserve and the ability of a plant to harden off. Dexter et al. (*3*) measured this hardening by electrolysis, and Newton and Brown (*18*) measured it by determining the bound water, stating that hydrophilic colloids bind water and increase the concentration of aqueous solutions and that factors affecting drought resistance include those concerned in absorption, transpiration, and wilt endurance. Newton et al. (*19*) also stated that moisture content is less in hardy varieties and that the resulting concentration of colloids and sugars in all fluids increases the resistance to freezing.

From this brief review of literature, it is evident that a better understanding of the carbohydrate metabolism and translocation with-

in the alfalfa plant is necessary to correlate it with the hardening processes and controlled cutting practices.

## METHODS OF EXPERIMENTATION

### OUTLINE OF EXPERIMENTS

The experiments were designed to show the effect of fall cuttings on crown-bud development and to correlate these cuttings with the food reserve storage and cold resistance of the roots and buds.

In earlier tests the writer (*6*, *7*) showed that fall cuttings of alfalfa more than any other affected the ability of the less winter-hardy varieties to maintain stands and also that these cuttings exerted a decided effect on the yield of hay from the first cutting of the following season.

In 1935 an experiment was begun in which cuttings were made from three replications, as shown in table 1. Particular attention was given to the effect of the number of days in the fall growing period. The later time-of-cutting experiments mentioned in the report follow this outline, except that the first three cuttings were all made at the same time and by regulating the time of the fourth and fifth cuttings various lengths of fall growing periods were obtained. Data on the crown buds and roots were taken on 100 plants dug from each plot. The following determinations were made and recorded: For crown buds, number, length, dry weight, percentage of carbohydrates and of total nitrogen; for roots, percentage of carbohydrates and of total nitrogen.

TABLE 1.—*Outline of experiments showing summer treatment by cuttings, date of last cutting, and fall treatment*

| Plot No. | Summer treatment by cuttings | | | | | Date of last cutting | Fall treatment |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| 1 | [1] B | B | B | B | B | Oct. 8 | Cut Oct. 8. |
| | B | B | B | B | | Aug. 26 | Uncut. |
| 2 | B | B | B | B | | do | Cut Oct. 8. |
| | B | B | B | B | | do | Uncut. |
| 3 | B | B | B | [2] FB | | Sept. 4 | Cut Oct. 8. |
| | B | B | B | FB | | do | Uncut. |
| 4 | B | B | FB | FB | | Sept. 14 | Cut Oct. 8. |
| | B | B | FB | FB | | do | Uncut. |
| 5 | B | FB | FB | FB | | Sept. 28 | Cut Oct. 8. |
| | B | FB | FB | FB | | do | Uncut. |

[1] B = bud. [2] FB = full bloom.

In 1937 a new experiment was started on a field previously seeded to Kansas Common and Ladak alfalfa. The object of this experiment was to verify the conclusions reached in the earlier work and to determine whether there was a varietal difference in the development of resistance to cold. In addition to the data taken as in the previous experiment, determinations of bound water, specific conductance of exosmosed material, total water, and dry weight were made on the crown-bud material.

### FIELD SAMPLING

All the top growth was removed from the root samples and discarded, the new crown buds were picked from the crowns, and the roots were

trimmed to 8-inch lengths, including the closely clipped crown. The fresh crown-bud material was used for determinations of bound water and specific conductance,extreme care being taken to minimize the loss of water while the samples were being prepared. That part of each sample to be used for chemical analysis was immediately oven-dried at 98° C. The individual pieces of buds were small, allowing rapid heat penetration and thus stopping all enzymatic action. The root samples for chemical analyses were cut into ½-inch lengths, placed in 95-percent alcohol, sealed, and stored until analyzed.

In preparing the roots for analysis the alcohol was drained off and the root material allowed to air-dry, after which it was coarsely ground, returned to the original alcohol, and mixed well. The ground material absorbed the alcohol, thus reincorporating the alcohol-soluble carbohydrates. The alcohol was allowed to evaporate and the dried material was reground until approximately 95 percent would pass through a ½-mm. sieve. This material was used for analyses of carbohydrates and total nitrogen.

### LABORATORY DETERMINATIONS

Results for all the analytical data are reported on a dry-weight basis.

The carbohydrate determinations were made from 2-gm. samples of the finely ground root and crown-bud material, and the reducing power of aliquot portions was determined in duplicate. The carbohydrate separates were determined by the cuprous titration method of Shaffer and Hartmann (*23*), and the amounts were calculated as dextrose from the Munson-Walker tables (*1*). Separation was accomplished by heating at approximately 80° C., with 90-percent alcohol, on a sand bath for 1½ hours. One aliquot of the filtrate was used to determine reducing sugars, and a second aliquot was hydrolyzed for three-quarters of an hour with 2½-percent hydrochloric acid for total sugars. The dextrins were separated from the residue with cold 10-percent alcohol, and an aliquot was hydrolyzed with 10 cc. of hydrochloric acid (1.19) for 2½ hours. The residue was washed into a beaker with hot water, boiled for 2 minutes, cooled, digested with saliva at 38° for 14 hours, filtered, and an aliquot portion hydrolyzed with 10 cc. of hydrochloric acid (1.19) for 2½ hours for starch. The residue was then hydrolyzed with 100 cc. of 1+20 hydrochloric acid for hemicelluloses.

Bound water was determined, by the dilatometer method as described by Rosa (*22*), on duplicate 10-gm. samples of the fresh crown-bud material. Petroleum ether was placed in the dilatometer with the samples, care being taken to exclude all air. The cooling was done by placing the filled dilatometers in calorimeters filled with cracked ice and salt.

Specific conductance determinations were made on duplicate 3-gm. samples frozen for 2 hours at −6° to −8° C. and exosmosed 4 hours in 60 cc. of distilled water. The specific conductance was reported in mhos, determined by the use of Student's potentiometer as a conductivity bridge. Dexter et al. (*3*) described this method and used it to measure the degree of cold resistance in plant material.

## EXPERIMENTAL RESULTS

### CROWN-BUD DEVELOPMENT

The growth and development of the crown buds of alfalfa were traced by taking samples from the experiment outlined in table 1. Samples were collected at intervals of 10 to 30 days from September 1935 to January 1936. The data covering the 1936 season are presented in figure 1 as averages for the dry weight of buds from plots 1 to 5 inclusive. These data show that the buds on the plants during the summer months are very few and small, but that they begin to increase in both number and size in September and continue to do so into November.

Since the buds that develop in the fall produce the next season's hay crop, conditions that favor the formation of numerous strong, healthy buds in the fall may aid in producing a larger hay yield the following season (*7*, *12*).

In the vicinity of Manhattan, Kans., top growth of alfalfa stops increasing in quantity about October 5 to 10. From that time until

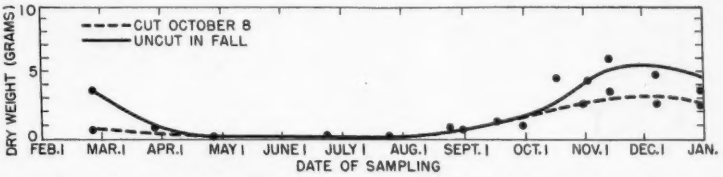


FIGURE 1.—Dry weight of crown buds from 100 alfalfa plants as affected by the fall cutting treatment outlined in table 1.

killing frosts occur, the plant continues to manufacture food and store it in the roots, a peak in storage being reached sometime in November.

The actual amounts of crown buds taken from 100 plants on plots 1 to 5 are shown in figure 2. The August 26 samples (fig. 2, *A*) were taken at about the time the buds began to develop, and the November 17 samples (fig. 2, *B* and *C*) were taken after all top growth had been stopped by freezing. The *B* samples were from the plots cut October 8 and the *C* samples from the uncut plots (table 1).

### FOOD RESERVES

The fluctuations in the reserves of alfalfa roots throughout the growing season and the effect of the length of the fall growing period on the total amount of food reserves stored for winter use have already been reported (*7*). The cutting practices that were favorable to high plant-food storage were also found to be favorable to crown-bud development.

The present experiment was originally planned to determine the effect of fall cutting treatments on the processes that harden Kansas Common and Ladak alfalfa to cold. Because of drought in the falls of 1937 to 1940, however, the amount of fall growth was so affected that no significant differences due to cutting treatments were found either in the top growth or in the food reserves. The severity of the drought in the fall of 1939 made sampling impracticable. Since the varietal differences between Kansas Common and Ladak were slight,

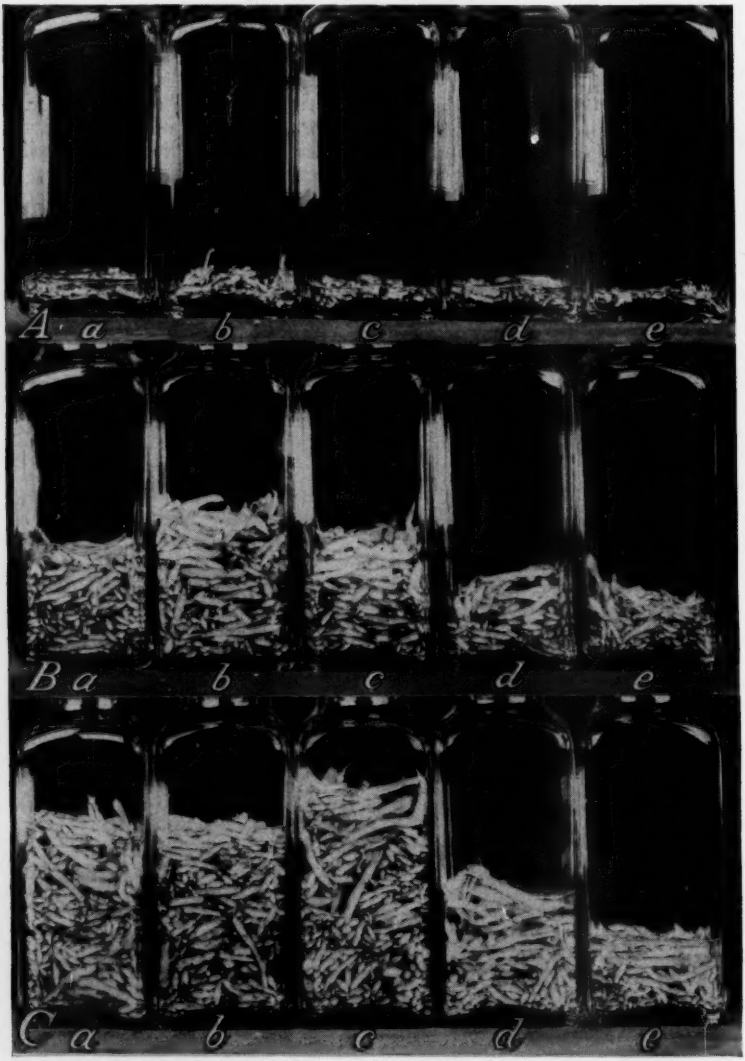


FIGURE 2.—Effect of cutting practices on development of alfalfa crown buds from plots 1 to 5 (*a-e*), as outlined in table 1: *A*, Samples taken at about the time the buds began to develop; *B* and *C*, samples taken November 17, after all top growth had been stopped by freezing, (*B*) from plots cut October 8 and (*C*) from uncut plots.

the data from the carbohydrate analyses for the two varieties were averaged (table 2). However, there was some indication that the hydrolysis of the stored starch to sugars in the roots during the hardening period was more complete in Ladak than in Kansas Common.

The data for total carbohydrates, starch, and total sugars are plotted as the deviation from the mean at the dates sampled (fig. 3). Each dot represents the average of eight field samples taken on approxi-

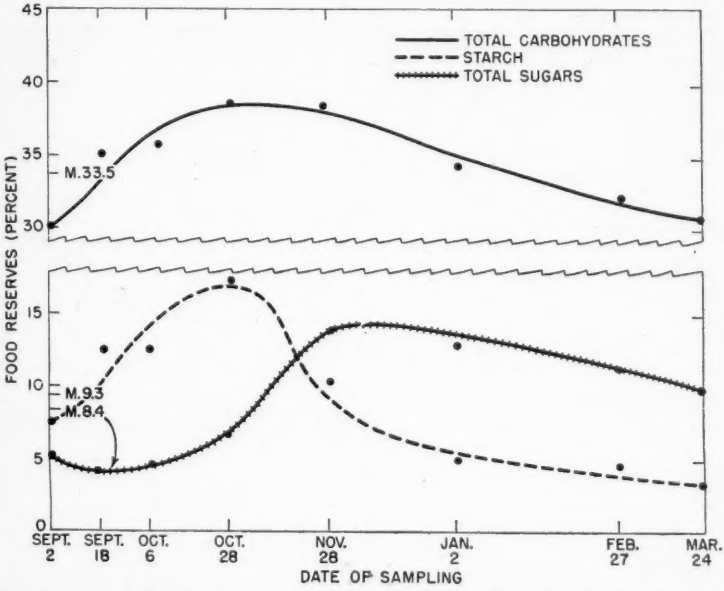


FIGURE 3.—The trend of plant food reserves in alfalfa roots, plotted as deviations from the mean.

mately the dates indicated in each of the years 1937, 1938, and 1940. Only total carbohydrates, starch, and total sugars are plotted, as the other determinations were more or less constant throughout the season (table 2). In 1937 hemicellulose and dextrin were not separated from the starch. In figuring the deviation for starch that year, it was assumed that all the fluctuations that took place were in the starch. No doubt there is some error in this assumption, but, as indicated by the 1938 and 1940 data, the fluctuation in starch was by far the greater, and by comparison the changes in hemicellulose and dextrins were not large enough to be important.

Most of the carbohydrates found in the crown buds of the alfalfa plant are probably translocated from the roots, as the buds are not storage organs.

The analytical data reported for the crown buds (table 2) were obtained from the same plants that were used for the root analysis. In figure 4 the percentages of total carbohydrates, starch, and total sugars are plotted as the deviation from the mean.

TABLE 2.—*Carbohydrates and nitrogen in roots and crown buds of alfalfa plants sampled at different dates*

PERCENTAGE OF CARBOHYDRATES IN ROOTS

| Constituent and year | Date of sampling | | | | | | | | Average |
|---|---|---|---|---|---|---|---|---|---|
| | Sept. 2 | Sept. 18 | Oct. 6 | Oct. 28 | Nov. 28 | Jan. 2 | Feb. 27 | Mar. 24 | |
| Total carbohydrates: | | | | | | | | | |
| 1937–38 | 28.70 | 26.55 | 33.75 | 39.20 | 39.05 | 34.10 | ..... | 28.75 | 32.87 |
| 1938–39 | 29.45 | 35.15 | 31.85 | 37.00 | 37.85 | 35.40 | 32.95 | 31.50 | 33.89 |
| 1940–41 | ..... | 28.80 | 37.70 | ..... | 38.55 | 33.20 | ..... | ..... | 34.56 |
| Starch: | | | | | | | | | |
| 1937–38 [1] | 25.80 | 24.50 | 30.75 | 34.50 | 29.20 | 20.80 | ..... | 10.05 | 25.09 |
| 1938–39 | 6.82 | 16.53 | 12.46 | 18.18 | 8.93 | 3.94 | 5.49 | 5.33 | 9.71 |
| 1940–41 | ..... | 8.18 | 12.07 | ..... | 7.55 | 6.88 | ..... | ..... | 8.67 |
| Total sugars: | | | | | | | | | |
| 1937–38 | 2.92 | 2.07 | 3.05 | 4.80 | 9.85 | 13.20 | ..... | 9.70 | 6.51 |
| 1938–39 | 7.71 | 4.74 | 5.59 | 7.08 | 14.87 | 14.76 | 12.68 | 9.68 | 9.64 |
| 1940–41 | ..... | 4.71 | 6.53 | ..... | 12.51 | 9.43 | ..... | ..... | 8.30 |
| Hemicellulose: | | | | | | | | | |
| 1938–39 | 14.05 | 12.60 | 13.30 | 11.80 | 11.90 | 12.25 | 13.75 | 15.60 | 13.16 |
| 1940–41 | ..... | 13.20 | 13.65 | ..... | 12.65 | 13.05 | ..... | ..... | 13.14 |
| Reducing sugars: | | | | | | | | | |
| 1937–38 | 1.75 | 1.92 | 2.23 | 3.86 | 4.03 | 8.65 | ..... | 4.47 | 3.84 |
| 1938–39 | 6.36 | 3.88 | 3.27 | 3.83 | 4.36 | 5.73 | 2.71 | 3.81 | 4.24 |
| 1940–41 | ..... | 3.49 | 4.41 | ..... | 4.54 | 2.92 | ..... | ..... | 3.84 |
| Dextrins: | | | | | | | | | |
| 1938–39 | .89 | .97 | .39 | 1.27 | 2.03 | 1.28 | .99 | .98 | 1.10 |
| 1940–41 | ..... | 1.16 | 1.04 | ..... | .91 | .87 | ..... | ..... | 1.00 |

PERCENTAGE OF CARBOHYDRATES IN CROWN BUDS

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Total carbohydrates: | | | | | | | | | |
| 1937–38 | 20.95 | 21.80 | 23.10 | 25.15 | 26.10 | 30.50 | ..... | 25.25 | 24.69 |
| 1938–39 | 16.65 | 19.30 | 19.85 | 22.65 | 29.65 | 32.25 | 29.95 | 20.85 | 23.89 |
| 1940–41 | ..... | 15.30 | 23.00 | ..... | 22.80 | 23.55 | ..... | ..... | 21.16 |
| Starch: | | | | | | | | | |
| 1937–38 [1] | 18.00 | 18.40 | 19.80 | 18.55 | 17.20 | 15.65 | ..... | 18.05 | 17.95 |
| 1938–39 | 2.15 | 3.43 | .72 | 1.40 | .33 | .50 | 1.01 | .55 | 1.26 |
| 1940–41 | ..... | 7.34 | 7.37 | ..... | 2.89 | 3.57 | ..... | ..... | 5.29 |
| Total sugars: | | | | | | | | | |
| 1937–38 | 3.41 | 3.35 | 3.31 | 6.59 | 8.90 | 14.83 | ..... | 7.33 | 6.82 |
| 1938–39 | 3.51 | 3.53 | 5.59 | 7.68 | 17.75 | 18.12 | 15.33 | 6.57 | 9.76 |
| 1940–41 | ..... | 4.77 | 3.68 | ..... | 8.51 | 9.07 | ..... | ..... | 6.51 |
| Hemicellulose: | | | | | | | | | |
| 1938–39 | 10.30 | 11.45 | 12.75 | 12.35 | 10.70 | 11.90 | 11.60 | 12.85 | 11.74 |
| 1940–41 | ..... | 1.76 | 10.47 | ..... | 10.59 | 7.83 | ..... | ..... | 7.66 |
| Reducing sugars: | | | | | | | | | |
| 1937–38 | 1.46 | 2.17 | 2.10 | 3.19 | 2.73 | 7.35 | ..... | 2.77 | 3.11 |
| 1938–39 | 1.50 | 1.64 | 1.58 | 2.65 | 2.15 | 2.05 | 2.48 | 1.66 | 1.96 |
| 1940–41 | ..... | 1.44 | 1.42 | ..... | .98 | 1.83 | ..... | ..... | 1.42 |
| Dextrins: | | | | | | | | | |
| 1938–39 | .56 | 1.10 | .77 | 1.15 | .87 | 1.72 | 1.97 | .92 | 1.13 |
| 1940–41 | ..... | [2] T | .46 | ..... | .78 | .89 | ..... | ..... | .71 |

PERCENTAGE OF NITROGEN IN ROOTS

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Total nitrogen: | | | | | | | | | |
| 1937–38 | 1.90 | 1.92 | 2.45 | 2.06 | 2.21 | 2.13 | ..... | 2.24 | 2.13 |
| 1938–39 | 1.38 | 1.87 | 1.70 | 1.92 | 2.06 | 2.12 | 2.29 | 2.30 | 1.96 |
| 1940–41 | ..... | 1.54 | 1.65 | ..... | 2.01 | 2.22 | ..... | ..... | 1.86 |

PERCENTAGE OF NITROGEN IN CROWN BUDS

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Total nitrogen: | | | | | | | | | |
| 1937–38 | 3.60 | 4.13 | 4.39 | 4.39 | 4.13 | 3.78 | ..... | 4.14 | 4.08 |
| 1938–39 | 3.85 | 4.45 | 4.14 | 4.56 | 4.30 | 3.69 | 4.09 | 4.04 | 4.14 |
| 1940–41 | ..... | 4.65 | 4.43 | ..... | 4.12 | 4.18 | ..... | ..... | 4.35 |

[1] During this season hemicellulose and dextrins were not separated from starch.
[2] T = trace.

The percentage data for total nitrogen are likewise given in table 2. From September to March, the general trend in the roots was upward, with an average of 1.98 percent. In the buds the average was 4.19 percent, the highest percentages being reached from late September through November.

## THE HARDENING PROCESS

### Changes in Carbohydrates

Changes in total carbohydrates are due largely to changes in the starch and total sugar content. In the roots, a large proportion of

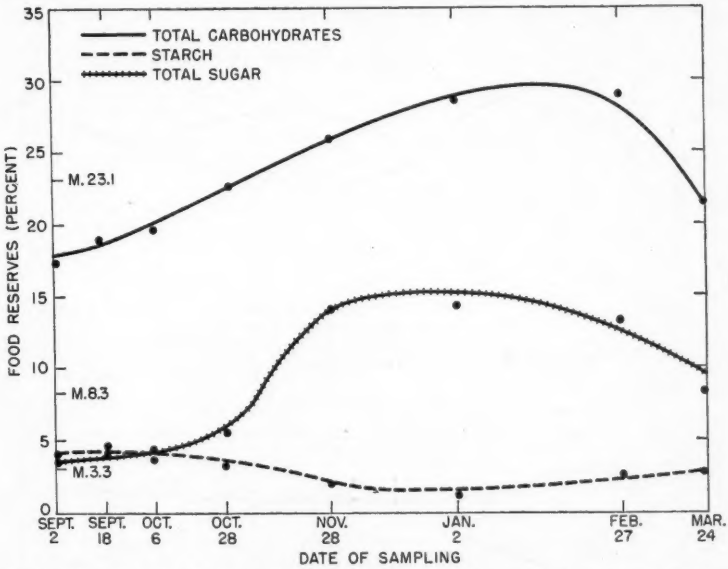


FIGURE 4.—Trend of plant-food reserves in alfalfa crown buds, plotted as deviations from the mean.

the total carbohydrates is stored as starch, whereas in the crown buds total sugars account for the largest proportion, and starch is present only in limited amounts (figs. 3 and 4). It is shown that storage in the roots continued until the latter part of October, when starch reached its maximum concentration. After that time starch decreased and total sugars increased, reaching a maximum in the roots during December. The maximum concentration of total sugars in the crown buds occurred during December and January and coincided with a rapid decrease of starch in the roots. Since little starch was present in the crown buds during the late fall and winter, the increase in sugar concentration was probably due to translocation from the root or crown tissue to the buds. Hydrolysis of starch and translocation of sugar occurred during the period when the plant was increasing in cold hardiness, and appeared to be a factor associated with the hardening process.

### Changes in Nitrogen

The total nitrogen content of the crown buds was found to average more than 2 percent higher than that of the roots, thus assuring sufficient nitrogen for the formation of proteins, which are largely colloidal in nature.

### Other Physiological Changes

Changes in dry weight, free water, bound water, and specific conductance of various plant parts of crop plants, including alfalfa roots, have been reported by a number of investigators. So far as the writer has been able to determine, however, no one has reported work on the crown buds of alfalfa and their relation to the storage organs in the utilization of stored carbohydrate and nitrogen reserves during

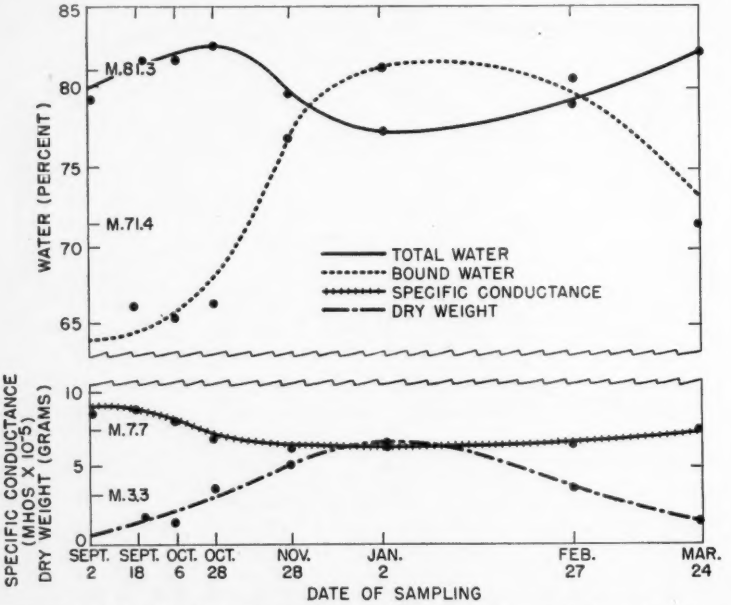


Figure 5.—Physiological changes in alfalfa crown buds, plotted as deviations from the mean.

the process of hardening to cold. Data here presented (table 3; fig. 5) indicate an increase in dry weight of the buds from September to January, after which the dry weight gradually decreased. Total water increased through the early fall, began to decrease the latter part of October, and reached a minimum in January, at a time when the buds exhibit greatest resistance to cold, as indicated by minimum values in specific conductance. The trend of the curve for bound water, calculated as percentage of total water, is opposite to that of total water, and there are no reverse changes in the early fall, as in the case of total water. The rapid increase in the percentage of bound water from October 6 to January 2 coincides with an increase in

cold resistance and with an increase in sugar. From table 3 it may be shown that approximately 20 percent of the total water from November to March remained as free water.

TABLE 3.—*Some physiological changes in crown buds of alfalfa plants at different dates of sampling*

| Item and year | Dry weight, amount of water, and specific conductance in samples taken on indicated date | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sept. 2 | Sept. 18 | Oct. 6 | Oct. 28 | Nov. 28 | Jan. 2 | Feb. 27 | Mar. 24 | Average |
| Dry weight: | *Grams* | *Grams* | *Grams* | *Grams* | *Grams* | *Grams* | *Grams* | *Grams* | *Grams* |
| 1937–38 | 0.82 | 1.62 | 2.73 | 3.24 | 2.94 | 3.62 | ------- | 1.79 | 2.39 |
| 1938–39 | .93 | 2.74 | 2.28 | 4.45 | 7.09 | 9.86 | 4.86 | 2.06 | 4.28 |
| Total water: | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* |
| 1937–38 | 76.9 | 81.3 | 81.4 | 83.7 | 80.0 | 76.4 | ------- | 81.21 | 80.1 |
| 1938–39 | 80.1 | 79.3 | 79.6 | 79.8 | 77.8 | 74.9 | 78.4 | 83.4 | 79.2 |
| 1940–41 | ------- | 84.9 | 84.7 | ------- | 82.2 | 81.5 | ------- | ------- | 83.3 |
| Bound water: [1] | | | | | | | | | |
| 1937–38 | 74.0 | 86.8 | 74.1 | 72.1 | 85.1 | 90.7 | ------- | 80.1 | 80.4 |
| 1938–39 | 64.5 | 63.2 | 62.3 | 72.9 | 83.6 | 89.4 | 84.1 | 75.2 | 74.4 |
| 1940–41 | ------- | 49.1 | 60.3 | ------- | 62.9 | 65.8 | ------- | ------- | 59.5 |
| Specific conductance: | *Mhos* ($\times 10^{-5}$) | *Mhos* ($\times 10^{-5}$) | *Mhos* ($\times 10^{-5}$) | *Mhos* ($\times 10^{-5}$) | *Mhos* ($\times 10^{-5}$) | *Mhos* ($\times 10^{-5}$) | *Mhos* ($\times 10^{-5}$) | *Mhos* ($\times 10^{-5}$) | *Mhos* ($\times 10^{-5}$) |
| 1937–38 | 7.2 | 10.0 | 8.0 | 7.8 | 6.3 | 5.7 | ------- | 8.6 | 7.7 |
| 1938–39 | 9.6 | 8.7 | 7.5 | 6.5 | 6.2 | 5.5 | 5.9 | 6.1 | 7.0 |
| 1940–41 | ------- | 7.8 | 9.5 | ------- | 7.1 | 9.2 | ------- | ------- | 8.4 |

[1] Calculated as percentage of total water.

Specific conductance has been used as an indicator of cold resistance in crop plants. Data shown in table 3 and figure 6 indicate the trend in

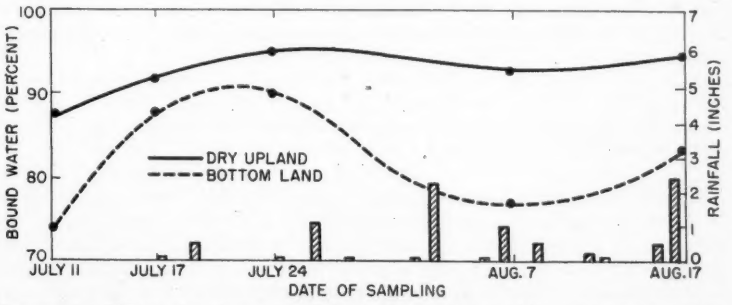


FIGURE 6.—Effect of moisture conditions on percentage of bound water in alfalfa roots.

the crown buds toward increasing hardiness, and correlate with the sugar content, total water, and bound water. All analytical data show more fluctuation between sampling dates in the fall than at any other time. This fluctuation may be caused by the rapid changes that often occur in growing conditions, such as alternate wet and dry or warm and cold periods during this season. When alfalfa is growing rapidly the carbohydrates in the roots decrease, whereas under conditions that limit vegetative growth, such as low soil moisture or low temperatures, photosynthesis continues, resulting in a rapid increase of reserves.

This fluctuation is reflected in all the data, indicating the influence of stored food on the other factors.

Environmental factors that tend to increase the dormancy of the alfalfa plant, regardless of the time in the growing season, will tend to increase its resistance to unfavorable conditions, bringing about physiological changes that makes it more resistant to cold in the winter (*2*, *7*, *13*) and to drought (*14*, *18*, *26*) in the summer. During the summer of 1939 data were obtained on the effect of moisture conditions on the bound water in alfalfa roots, and these are presented in figure 6. Observations had shown that there was a difference in the effect of available moisture on the growth of alfalfa tops in the two adjoining areas sampled. In June before the first sampling date, growing conditions were good, the rainfall of 7.3 inches for the month being fairly evenly distributed. Between July 2 and 17 (fig. 6) no rain fell, and the mean maximum temperature was 100° F. for the 15 days, causing the bound water to increase rapidly. After July 17, there were again light rains and until August 7 growing conditions were favorable on the bottom land because of the combined effect of the rainfall and the runoff from the upland. During this period the bound water decreased. Because of the rapid runoff, the plants on the upland received very little benefit from the rainfall. Between August 7 and 16 the weather was again hot and dry, and this condition was soon reflected in the bound-water content of the roots from the bottom land. The weather changes were not sufficient to exert much effect on the bound water in the roots of the upland samples.

## DISCUSSION

Earlier reports on work in this series of experiments (*6*, *7*) have shown that the carbohydrate content and the nitrogen content of the roots rise and fall with the development of new growth in the spring or after cutting. It has also been shown (*7*) that when top-growth conditions in the fall are favorable for high food reserves and for the development of numerous and vigorous crown buds, there is a more vigorous top growth the following spring and an increase in the yield of hay from the first cutting.

The physiological factors recorded in figure 5 suggest that the increase of carbohydrates in roots and buds and the high nitrogen content of the buds (table 2) are closely associated with the increase in bound water and the reduction in free water. It is definitely known that proteins are not formed in the plant without a supply of carbohydrates as a constituent, as well as a form of energy necessary for the reduction of nitrogen (*16*). During the hardening period, between October 6 and November 28, the rapid translocation of sugars from the roots increased the concentration of the cell sap in the buds, which tended to lower the percentage of total water. From October 6 to January 2 there was also a 20-percent increase in the amount of total water that became bound water. An additional factor in the reduction of free water was probably the high carbohydrate content in both the roots and the buds, which supplied the necessary energy for the reduction of the nitrogen, thus increasing the colloidal content of the buds.

SUMMARY

The time of fall cutting has been shown to have a direct influence on the physiological processes that take place in the alfalfa plant.

Fall cutting experiments conducted between 1935 and 1940 dealt directly with the processes of the alfalfa plant that influence the initiation and growth of the crown buds and their relation to cold resistance. The factors studied included food reserves, bound water, specific conductance of exosmosed material, total water, and dry matter.

Both the number and the development of the crown buds were influenced by the amount of fall top growth remaining on the plants during the fall, indicating the importance of making the last cutting early enough to allow at least 6 inches of top growth to develop before the beginning of the fall dormant period.

Cutting practices favorable to high food storage were also favorable to crown-bud development. Cutting practices are also closely correlated with the hardening processes, as shown by the amount of total water, bound water, and specific conductance.

The hardening period, as shown by these determinations, extends from approximately September 1 to December 1, the most rapid changes taking place between October 1 and December 1. Some changes indicate that increased resistance occurred as late as January 1. During the hardening period a rapid hydrolysis of starch, the principal form of plant food storage, occurred, with a subsequent translocation of the resulting sugar to the crown buds.

The average amount of total nitrogen in the roots was 1.98 percent, dry weight, and in the crown buds, 4.19 percent. The high nitrogen content of the crown buds may account in part for their high content of bound water.

The decrease in percentage of total water was associated with the increased concentration of sugars and the high nitrogen content of the buds, which resulted in the further binding of the free water with protoplasmic compounds.

The decrease in the amount of free water to approximately 20 percent of the total water and the increased concentration of sugars lowered the freezing point of the crown-bud tissue, as shown by the specific conductance test.

The data presented in these and earlier experiments indicate that food reserves are essential for developing cold resistance in alfalfa and from a practical standpoint can be influenced by proper cutting practices.

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# MUSTARD OILS IN CRUCIFERS AND THEIR RELATION TO RESISTANCE TO CLUBROOT[1]

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## INTRODUCTION

It has been shown by a number of workers (*7*, *23*, *30*, *31*)[3] that there exists within the Cruciferae a wide range of resistance and susceptibility to the clubroot organism (*Plasmodiophora brassicae* Wor.). After a rather extensive anatomical study of the root structure of resistant and susceptible varieties, Rochlin (*23*, *24*) showed that there is no basis for resistance to be found in the root morphology. She decided, because of an apparent correlation between mustard-oil content and resistance, that the mustard oil in certain varieties and species was responsible for their resistance to clubroot on the basis of the high toxicity of the isothiocyanates to micro-organisms (*14*, *6*, *22*, *33*). The correlation was indicated in a table listing a number of species and varieties of crucifers, the percentage of infected plants recorded for each by various workers, and the relative amount of mustard oils reported for the same forms. It should be pointed out that in many cases where mustard oils were listed as absent, a search of the literature has revealed reports of the identification of mustard oils in these species. Rochlin listed the absence of mustard oils in each of 3 varieties of cabbage (*Brassica oleracea* var. *capitata* L.) which she investigated. On the other hand, Grimme (*10*) reported the presence of mustard oils in each of 10 varieties of cabbage which he studied. Likewise, Rochlin listed the absence of mustard oils in *Sinapis arvensis*, but Rothea (*25*) reported mustard oils in this slant. The evidence which appeared to be the real basis for Rochlin's explanation consisted principally of an experiment in which the volatile mustard-oil content of a number of resistant and susceptible varieities was roughly estimated. This was done by noting the relative strength of the pungent odor arising from root-tissue macerates of resistant and susceptible plants. A correlation between the relative strength of the mustard-oil odor and resistance was reported.

In view of the conclusions reached by Rochlin, it was decided to conduct a biochemical study of the mustard-oil fraction from the root tissues of varieties and species resistant and susceptible to clubroot to

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[3] Italic numbers in parentheses refer to Literature Cited, p. 62.

determine whether the isothiocyanates were a factor in determining this resistance. The present paper is one of a series having to do with resistance to clubroot in certain forms of black mustard (*Brassica nigra* (L.) Koch) and turnip (*B. rapa* L.). The determination of the relative resistance to clubroot of these forms has been reported elsewhere (*30, 31*). Other species included for comparative study were white mustard (*B. alba* (L.) Boiss.), cabbage (*B. oleracea* var. *capitata* L.), and horseradish (*Armoracia rusticana* Gaertn.).

The first phase of this study represents a qualitative investigation of the mustard oils of resistant and susceptible forms to ascertain whether resistance could be ascribed to the presence of specific mustard oils. The second phase deals with the development of a chemical method of analysis for the estimation of mustard oils in plant tissue, and the application of this method to the quantitative determination of the mustard-oil content of resistant and susceptible root tissues. The third part is a study of the mustard-oil enzyme system in resistant and susceptible plants.

## EXPERIMENTAL RESULTS

### ISOLATION OF MUSTARD OILS

The general procedure used was first worked out on the cortical [4] tissue of mature fleshy roots of turnip. This scheme with some modifications was later applied to the root tissues of other crucifers.

Three kilograms of the fresh tissue, peeled from the roots, was ground to a pulp in a Nixtamal mill. The pulp was divided into four 750-gm. portions and each portion placed in a 5-liter steam distillation flask containing 1,500 ml. of water. A rapid exhaustive steam distillation was immediately carried out. Since the hydrolysis of the mustard-oil glycosides in root tissues occurs rather rapidly, distillation immediately after grinding usually gave slightly higher yields of mustard oil than distillation after a 3-hour period of maceration. After a 12-hour maceration period, yields were reduced to about one-fourth of the amount that could be obtained immediately after grinding.

Since the amount of plant tissue available for this study was limited, it was decided to purify the mustard-oil fraction by crystallization of the corresponding substituted thiourea rather than by fractional distillation of the very small amount of mustard oil that would be obtained from the steam distillation. Accordingly, the distillate was collected in 2-liter receivers containing 25 ml. of concentrated ammonium hydroxide. To prevent loss of the volatile mustard oils, the condenser was fitted with an adapter which dipped below the level of the liquid in the receiver. From 3 kg. of tissue, about 6–liters of distillate was collected. The distillate containing excess ammonium hydroxide was allowed to stand at room temperature for 12 hours to complete thiourea formation, and then extracted 12 hours with peroxide-free ethyl ether in a continuous liquid-liquid extractor.

The ether solution from 6 kg. of the cortical tissue of turnip roots was concentrated to about 100 ml., 200 ml. of 95-percent ethanol added, and the remaining ether removed by distillation. The light yellow alcoholic solution containing the thiourea fraction corresponding to the mustard oils in the distillate was treated with a small amount

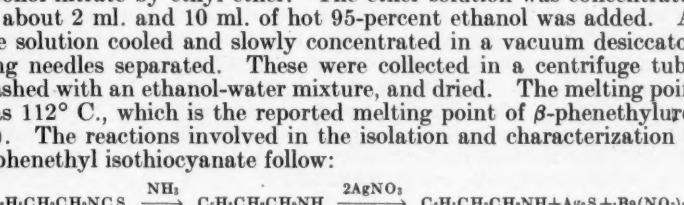
[4] The term "cortical" as used in this paper refers to the tissue of the turnip fleshy root obtained when an outer peel about 2 mm. in thickness was removed. Anatomically such tissue is not primary cortex but periderm, phloem, and possibly some secondary xylem

of carbon to remove some of the color. An excess of carbon was avoided since it might adsorb significant amounts of the thiourea fraction. The alcoholic filtrate was concentrated under reduced pressure to about 25 ml. and transferred to a crystallizing dish. The solution was then heated on a steam bath and hot water added to the first point of turbidity. On cooling and slowly removing the alcohol by concentrating in a vacuum desiccator, crystalline, diamond-shaped plates separated. This crude, water-insoluble thiourea fraction was collected by filtration in yields from 0.2 to 0.8 gm. depending on the variety used. This product was recrystallized from ethanol-water mixtures to a constant melting point, 136° to 137° C. Repeated attempts were made to isolate other thioureas from the mother liquors by extraction with different solvents, fractional crystallization, and high vacuum distillation, but only the one product could be obtained.

### CHARACTERIZATION OF MUSTARD OILS

The melting point could not distinguish between the thiourea obtained from *d*-secondary-butyl isothiocyanate, melting point 134° C., and that from β-phenethyl isothiocyanate, melting point 137° C. Both mustard oils have been isolated from crucifers (*27*). The mixed melting point with a synthetic sample of β-phenethyl thiourea, the elementary analysis, and conversion of the isolated thiourea to the corresponding urea were used to distinguish between these two possible thioureas. Accordingly, a portion of the thiourea isolated from each species was converted to the substituted urea. In this way two derivatives of mustard oil could be obtained from the same sample.

The conversion of the thiourea to the substituted urea was carried out by a modification of the method of Bertram and Walbaum (*3*). Twenty milligrams of the thiourea was dissolved in 10 ml. of ethanol and a slight excess of 0.1 N barium hydroxide added. A slight excess of an aqueous solution of silver nitrate was then added and the mixture heated on a steam bath for 1 hour. The silver sulfide was removed by filtration and the urea extracted from the aqueous alcohol filtrate by ethyl ether. The ether solution was concentrated to about 2 ml. and 10 ml. of hot 95-percent ethanol was added. As the solution cooled and slowly concentrated in a vacuum desiccator, long needles separated. These were collected in a centrifuge tube, washed with an ethanol-water mixture, and dried. The melting point was 112° C., which is the reported melting point of β-phenethylurea (*3*). The reactions involved in the isolation and characterization of β-phenethyl isothiocyanate follow:




*Analysis of β-phenethylthiourea (isolated)* $C_9H_{12}N_2S$

| | | | | |
|---|---|---|---|---|
| Calculated: | C–60.00 | H–6.66 | N–15.55 | S–17.77 |
| Found: | C–60.54, 60.57 | H–6.58, 6.70 | N–15.85, 15.74 | S–17.85, 17.72 |

SYNTHESIS OF MUSTARD OILS

For purposes of comparison with the material isolated from root tissues and for a study of its relative toxicity to fungi, a sample of β-phenethyl isothiocyanate was synthesized according to the following scheme:

$$C_6H_5CH_2Cl+NaCN+NaOH \longrightarrow C_6H_5CH_2CN+NaCl$$

$$C_6H_5CH_2CN+H_2 \xrightarrow[125°]{Ni} C_6H_5CH_2CH_2NH_2$$

$$C_6H_5CH_2CH_2NH_2+CS_2+KOH \longrightarrow C_6H_5CH_2CH_2NH+H_2O$$

$$\begin{array}{l} \\ \\ \\

root tissue of resistant crucifers (*23*, *24*), it was thought desirable to identify the mustard oils existing in the roots of representative varieties of different species, resistant and susceptible to clubroot.

It should be pointed out that the isolated yield of crystalline thiourea is not to be considered an exact representation of the quantity of mustard oil or mustard-oil glycoside present in the plant tissue. Loss of mustard oil may occur through hydrolysis or by reaction of the isothiocyanate group with other compounds in the tissue macerate during the distillation. The amount of other volatile oils accompanying the mustard-oil fraction fluctuated with the plant being studied. This variation made differing amounts of purification necessary for the isolation of crystalline thiourea. In some cases one or two recrystallizations yielded the pure thiourea. In other cases repeated recrystallization and decolorization with carbon or a high-vacuum distillation were necessary before the thiourea could be separated from traces of an accompanying yellow oil. Hence, losses during isolation are not proportionate or always comparable. Additions to turnip-tissue macerates of β-phenethyl isothiocyanate in amounts from one-half to three times that previously isolated indicated that about 50 percent of that added could be recovered as crystalline thiourea by this isolation method.

The mustard-oil fraction from the root tissue of the other crucifers was isolated as the substituted thiourea by the method described for turnip tissue, except that from 6 to 15 kg. of the whole, fresh roots were ground and subjected to the steam distillation. In all the varieties investigated in this study except horseradish, the mustard oil isolated was characterized as β-phenethyl isothiocyanate by (1) the melting point of the crystalline thiourea, 136° to 137° C., (2) the melting point of a mixture of the isolated thiourea with an authentic sample of β-phenethylthiourea, and (3) the melting point, 112° C., of the urea obtained from the isolated thiourea in the manner described for the material from turnip tissue.

With all forms studied repeated attempts were made to separate other mustard oils as their thioureas. But in only one case (horseradish) could a second mustard oil be obtained from root tissue. The small amount of total solids in the mother liquors and their low sulfur content would indicate that, if other mustard oils were present, they were to be found only in comparatively minute quantities. That these isolation methods were capable of detecting other mustard oils was shown by the recovery from horseradish tissue of a second thiourea, that corresponding to allyl isothiocyanate. When allyl isothiocyanate was added to turnip-tissue macerates in amounts approximately equal to the β-phenethyl isothiocyanate content of the tissue, approximately half of the added isothiocyanate could be recovered as allyl thiourea after removing the β-phenethylthiourea.

The species and varieties examined in this study and the amounts of mustard oil recovered as thiourea are given in table 1. β-phenethyl isothiocyanate was isolated and characterized from fresh and from dried turnip root tissue. This mustard oil was isolated from each of four strains of black mustard and from one strain each of cabbage, white mustard, and horseradish. Thus the same mustard oil has been shown to be the principal one present in the root tissues of both resistant and susceptible varieties of black mustard and turnip; in

TABLE 1.—*The mustard oils isolated from roots of various crucifers differing in their resistance to clubroot*

| Species | Variety or collection | Resistant or susceptible to clubroot | Amount of thiourea obtained per kilogram of fresh tissue | |
|---|---|---|---|---|
| | | | β-phenethyl | Allyl |
| | | | *Milligrams* | *Milligrams* |
| Turnip (*Brassica rapa*) | Purple Top Milan | Resistant | 245 | 0 |
| | Shogoin | Susceptible | 85 | 0 |
| Black mustard (*Brassica nigra*) | 3 | Resistant | 33 | 0 |
| | 10 | do | 30 | 0 |
| | 8 | Susceptible | 28 | 0 |
| White mustard (*Brassica alba*) | 14 | do | 39 | 0 |
| | 15 | do | 54 | 0 |
| Cabbage (*Brassica oleracea capitata*). | Jersey Queen | do | 104 | 0 |
| Horseradish (*Armoracia rusticana*) | | Resistant | 137 | 100 |

cabbage and white mustard root tissue, where all varieties known are susceptible; in the root tissue of horseradish, which is usually reported as highly resistant; and in radish root tissue. It is evident, therefore, that this mustard oil is characteristic of root tissues of many crucifers.

In the case of horseradish, whole, mature roots were ground in a hammer mill and then covered with water for 1 hour before distillation. The isolation of β-phenethylthiourea from the distillate was carried out as described for turnip tissue. In addition, allylthiourea was isolated from the aqueous mother liquors after removal of the β-phenthylthiourea. These mother liquors were concentrated to dryness, the residue was dissolved in 100 ml. of hot absolute ethanol, and the solution was decolorized with carbon. On cooling and slowly concentrating in a vacuum desiccator, crystals separated. After recrystallization their melting point was 74° C. This coincides with the melting point of allylthiourea, and a mixed melting point with an authentic sample of allylthiourea showed no depression.

The isolation of β-phenethyl isothiocyanate from one variety of turnip root tissue has been previously reported by Kuntze (*15*). The isolation of this mustard oil and allyl iosthiocyanate from horseradish root tissue has been reported by Heiduschka and Zwergal (*12*) and by Pietschmann (*20*). Bertram and Walbaum (*3*) found the former in root tissue mignonette (*Reseda odorata* L.).

### QUANTITATIVE ESTIMATION OF MUSTARD OILS IN PLANT TISSUE

Studies of the quantitative methods for the determination of mustard oils were undertaken to develop analytical methods that could be applied to (1) the determination of the total mustard-oil content of root-tissue macerates as an estimation of the potential mustard-oil content of the tissue, and (2) a study of the rate of hydrolysis of a mustard-oil glycoside substrate by tissue macerates as an estimation of the mustard-oil enzyme activity of the tissue. It was thought that if mustard oils were concerned with resistance to clubroot, either the rate of their release by enzymatic hydrolysis when infection began or the total amount available might become limiting factors.

The literature gives various methods for the determination of mustard oils. Most of these have been devised to estimate allyl isothiocyanate in mustard seed or the mustard oil of commerce. The

estimation of the small amounts of mustard oil present in root-tissue macerates presented some difficulties that prevented the direct application of any single described method to this problem. In the development of the analytical scheme used in this study, a number of the methods of mustard-oil analysis that have been described in the literature were studied (*2, 4, 8, 11, 16, 17, 20, 29*), purified samples of β-phenethyl and allyl isothiocyanates being used. The final scheme made use of some of the steps in these methods, together with some new techniques. The individual experiments on which each of the steps in these analytical schemes was based will not be described in detail. It will suffice to state that each operation was first studied separately to insure that it could be carried out quantitatively. Then the individual operations were combined and the entire scheme checked against purified mustard oils and a mustard-oil glycoside.

The method finally adopted for the estimation of the total mustard-oil content of root tissue is based on the following steps: (1) Removal of the mustard oil from the tissue macerate by steam distillation. (2) conversion to the thiourea, (3) extraction of the thiourea with ether, and (4) estimation of the thiourea by measuring the silver consumed in precipitating the sulfur by the Volhard titration. It is recognized that this procedure may not give an absolute measure of the mustard-oil glycoside content of the tissue because the enzymatic hydrolysis does not go to completion. Furthermore, losses of mustard oil may occur before or during the distillation by hydrolysis of the isothiocyanates, by condensation with other plant constituents, or by the action of a mustard oil enzyme system. However, it is believed that although the results may not be absolute, they will serve in comparing the total mustard oil or mustard-oil glycoside content of resistant and susceptible crucifers.

Mature turnips were harvested and the fleshy roots washed and peeled. The cortical tissue was sliced and mixed. A sample was taken for the moisture determination. The sliced tissue was then ground to a pulp in a Nixtamal mill. Four 750-gm. portions (3 kg.) were weighed into 5-liter steam-distillation flasks and subjected immediately to exhaustive steam distillation. The condensers were fitted with an adapter and outlet which reached below the level of the liquid in the receiver. The receivers contained 50 ml. of concentrated ammonium hydroxide in 200 ml. of water. Six liters of distillate was collected. The distillate appeared clear during the latter half of the distillation. The distillates were combined, stirred for 2 hours, and allowed to stand 12 hours to complete conversion of the mustard oil to the corresponding thiourea. The thiourea was then extracted from this aqueous solution with peroxide-free ethyl ether in a continuous liquid-liquid extractor. The ether solution was concentrated to about 50 ml. and transferred to a 500-ml. volumetric flask, using 25 ml. of ethanol and about 300 ml. of water to wash the contents of the boiling flask into the volumetric flask. The flask was then heated to about 60° C. to remove the remaining traces of ether. Standard 0.1 N silver nitrate solution (100 ml.) was then added and the contents mixed by swirling the flask. After the reaction mixture had stood 24 hours to complete the sulfide precipitation, the contents of the flask were brought to volume with water, mixed, and the precipitated silver sulfide filtered off through a dry filter paper.

One hundred-milliliter aliquots of the filtrate were acidified with 10 ml. of 3 N nitric acid and titrated with standard 0.1 N ammonium thiocyanate solution ferric-ammonium alum being used as an indicator. The mustard-oil content of the tissue was calculated as β-phenethyl isothiocyanate from the amount of silver removed as silver sulfide.

Mustard roots were analyzed in a similar manner. The fresh, mature roots were dug, washed, and the whole roots ground in a Wiley mill. Because of the limited amount of mustard-root tissue available, the sample size was limited to about 1 kg. Distillation and mustard-oil estimation were carried out as described for turnip tissue.

### MUSTARD-OIL CONTENT IN RELATION TO DISEASE RESISTANCE

The results secured with resistant and susceptible turnip and black mustard are given in table 2. It will be seen that no correlation was

TABLE 2.—*Mustard-oil content of root tissue of resistant and susceptible turnip and black mustard plants*

| Species | Variety or collection | Resistance | Mustard oil [1] |
|---|---|---|---|
| | | | *Percent* |
| Turnip | Snowball | Highly resistant | 0.019 |
| | White Milan | do | .015 |
| | Purple Top White Globe | do | .021 |
| | Purple Top Milan | do | .024 |
| | Cowhorn | Moderately resistant | .020 |
| | Shogoin | Very susceptible | .017 |
| Black mustard | 3 | Resistant | .010 |
| | 14 | Susceptible | .016 |

[1] Expressed as β-phenethyl isothiocyanate calculated on fresh weight.

found between the resistance to clubroot of the strains from which the tissue samples were collected and the chemical estimation of mustard oil.

In studies parallel with these, Pryor (*21*) had shown that when resistant mustard and turnip were grown in sand-nutrient cultures in which sulfur was omitted, the presence of mustard oils in the leaves could not be detected by taste, but resistance to clubroot was nevertheless not broken down. It was of interest, therefore, to study the mustard-oil content of resistant and susceptible plants grown with and without sulfur in the nutrient. Stems and leaves were frozen with solid carbon dioxide, ground while frozen, and heated to 37° C. in water for one-half hour before distillation. The distillate was collected in an excess of ammonium hydroxide. After conversion to the thiourea was complete, the distillate was extracted with ether and the analyses carried out in a manner similar to that used with root tissue except that smaller samples and less silver nitrate were used.

The results are given in table 3. It will be seen that in all cases the mustard-oil content was reduced to a trace or a very small amount as compared with that in plants grown in a standard nutrient. Nevertheless, the resistant plants remained resistant, and the susceptible strains were heavily infected, according to Pryor (*21*).

It is clearly evident that the total mustard-oil content of the crucifers studied was not related to their resistance or susceptibility

to clubroot. Furthermore, when the oil content was raised or lowered through nutrition, no measurable effect on infection and resistance was to be noted.

TABLE 3.—*Mustard-oil content of the stems and leaves of plants grown in sand-nutrient culture with and without sulfur*

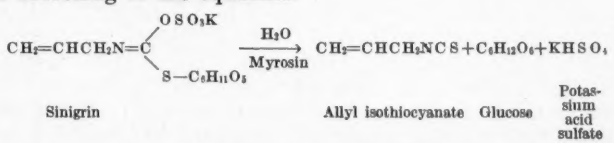
| Species | Variety or collection | Resistance | Mustard oil [1] determined in plants grown— | |
|---|---|---|---|---|
| | | | With sulfur | Without sulfur |
| | | | *Percent* | *Percent* |
| Black mustard | 10 | Resistant | 0.026 | 0.005 |
| | 14 | Susceptible | .043 | .002 |
| Turnip | Purple Top Milan | Resistant | .003 | [2] |
| | Shogoin | Susceptible | .002 | [2] |
| Cabbage | Jersey Queen | do | .004 | .001 |

[1] Expressed as β-phenethyl isothiocyanate calculated on fresh weight.
[2] Trace.

## MYROSIN ACTIVITY OF ROOT TISSUE

### The Mustard-Oil Enzyme System

Mustard oils occur in the plant cells in a conjugated form as glycosides. The best known example of a mustard-oil glycoside is sinigrin (potassium myronate), which on enzymatic hydrolysis yields 1 mole of allyl isothiocyanate, *d*(+)-glucose, and potassium acid sulfate according to the equation:




The enzyme system that effects this hydrolysis is called myrosin. It has been demonstrated to be widely distributed in the tissues of the crucifers and in some members of other plant families (*27*). Neuberg (*18*), and Neuberg and Wagner (*19*) have shown myrosin to consist of two enzymes, a sulfatase which splits off potassium acid sulfate, and a thioglucosidase which splits the thioglycoside linkage. Sandberg and Holly (*26*) have shown by glucose determinations that hydrolysis by thioglucosidase proceeds only to a definite equilibrium value (66 percent), but sulfate determinations showed complete hydrolysis of the potassium acid sulfate. The equilibrium value has not previously been determined by mustard-oil analysis.

The relative myrosin activity of plant tissue could be determined by measuring the amount of hydrolysis of a sinigrin substrate effected by a tissue macerate in a given time. To obtain the rate of mustard-oil liberation by the mustard-oil enzyme systems of resistant and susceptible plants, it was thought best to determine the allyl isothiocyanate liberated. For such a study it is necessary to allow the hydrolysis to proceed for a given time, then stop the reaction immediately without altering the concentration of the hydrolytic products, and determine one of the products (allyl isothiocyanate) by a chemical method that is not affected by the unhydrolyzed sinigrin or other products from the tissue macerate.

### The Determination of Allyl Isothiocyanate From the Hydrolysis of Sinigrin

To develop a chemical method to serve this purpose, a comparison was first made of some of the methods that have been described for allyl isothiocyanate analysis of black mustard seed. Morvillez and Meesemeacker (*17*) have suggested the use of the absorption of iodine by the seed distillate as an estimation of allyl isothiocyanate, the absorption taking place at the double bond of the allyl group. Meesemeacker and Boivin (*16*) have used the iodine method directly on filtrates from seed macerates and thus have eliminated the tedious steam distillation. Although this procedure has been questioned (*11*), it was considered feasible and practical for use in these studies since it would permit the rapid determination of the allyl isothiocyanate immediately after stopping the enzymatic hydrolysis. The iodine absorption procedure as modified by Viebeck and Brecker (*29*) was found to be most practical and to give accurate and reproducible results when applied to the analysis of purified allyl isothiocyanate. The allyl isothiocyanate is converted to allylthiourea by excess ammonium hydroxide and the iodine absorption by the allyl group measured.

When this procedure was applied to the analysis of the hydrolytic products of a pure sinigrin substrate by a purified myrosin preparation, widely variable results were obtained which were much higher than the equilibrium value reported by Sandberg and Holly (*26*). These results suggested iodine absorption by the unhydrolyzed sinigrin, but when sinigrin was treated with iodine under these conditions, no iodine absorption could be observed. When sinigrin was first treated with excess ammonium hydroxide, as is done in these methods to convert allyl isothiocyanate to thiourea, and then treated with iodine, large but variable iodine absorptions were observed. When sinigrin was treated with a stronger base (0.1 N sodium hydroxide), a quantitative iodine absorption was obtained, and the solution gave a strong test for sulfate ions. No mustard oil could be detected in the alkaline solution.

It was necessary, therefore, to separate the allylthiourea from the hydrolysis mixture before estimation of the allylthiourea by iodine absorption. This was accomplished by ether extraction of the thiourea from the hydrolysis mixture in a continuous extractor after treatment with excess ammonium hydroxide. Iodine absorption then gave reproducible values which indicated that at equilibrium the myrosin had split off about 80 percent of the allyl isothiocyanate. The curve in figure 1 was obtained by measuring the allyl isothiocyanate released from a buffered (pH 7.2) solution of sinigrin by a fixed amount of a purified myrosin preparation. The sinigrin was prepared according to the method of Herissey and Boivin (*13*) and the myrosin by the method of Braecke (*5*).

### Determination of Myrosin Activity of Root Tissue

Determinations of the relative myrosin activity of the root tissue of resistant and susceptible varieties of turnip and black mustard were made. With the turnip, separate determinations were made on the outer tissue, obtained by carefully peeling the fleshy root, and on the

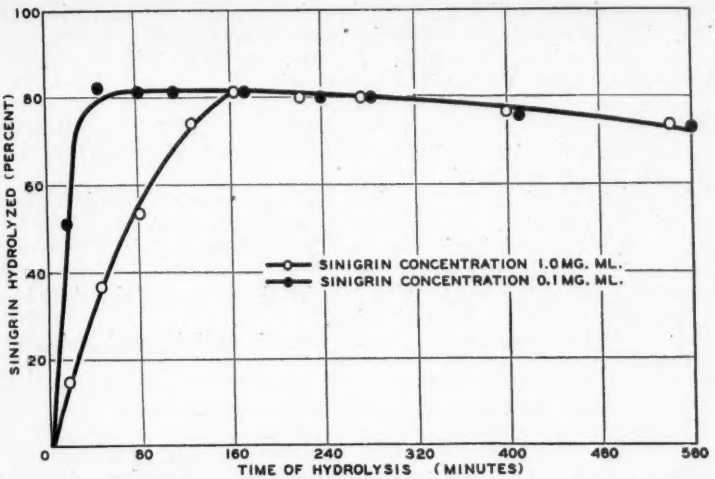



FIGURE 1.—The enzymatic hydrolysis of sinigrin as measured by allyl isothiocyanate formation.

remaining inner portion of the root. The fresh turnips were peeled, and 45 gm. of the outer and 90 gm. of the inner tissue were taken for study. The samples were sliced, then frozen in solid carbon dioxide and ground through a fine meat grinder while frozen. The tissue was allowed to thaw and ground further in a mortar with a little sand. This suspension was shaken for 12 hours with 75 ml. of a phosphate buffer at pH 7.2 and filtered.

Ten milliliter of the filtrate which would contain the water-soluble myrosin was added to 10 milliliter of a 1-percent sinigrin solution in phosphate buffer at pH 7.2. This mixture was maintained at 45° C. for 165 minutes. Ten milliliter of concentrated ammonium hydroxide was then added to stop the hydrolysis and the mixture heated for 1 hour at 70° C. to convert the mustard oil to the thiourea. The thiourea was extracted from the hydrolysis mixture by a 4-hour continuous extraction with ethyl ether. Twenty milliliter of water was added to the flask containing the ether extract and the ether evaporated by heating on a steam bath to about 60° C. The aqueous solution was neutralized to methyl red with dilute nitric acid and transferred to a 250-milliliter iodine titration flask. Enough distilled water was used in washing the extraction flask to bring the volume to about 100 milliliters. Then 10 milliliters of a solution of equal parts of 0.1 N HCl and glacial acetic acid was added. Five milliliters of 0.02 N standard iodine solution was added from a pipette, the flask stoppered, swirled, and allowed to stand in the dark for 30 minutes. The unused iodine was then determined by titration with 0.01 N standard thiosulfate. Blank analyses were made on solutions containing no added sinigrin to correct for iodine absorption by materials from the tissue extract. This absorption proved to be very low.

The determination of the myrosin activity of mustard roots was carried out in a similar manner, except that the fibrous nature of the

roots necessitated a different technique in preparing the myrosin solution. The mustard roots were dried before a fan at 25° C., then ground to fine powder in a Wiley mill. A 2-gm. sample of this ground root tissue was ground further in a mortar with sand, then extracted for 12 hours by shaking with 50 milliliters of phosphate buffer. Ten milliliters of the filtrate was added to the buffered sinigrin substrate and the determination carried out as for the turnip tissue, except that the time for the enzymatic hydrolysis was changed.

The results obtained with a resistant and a susceptible turnip variety and with two resistant and two susceptible black mustards are shown in table 4. The activity is expressed as milligram of sinigrin hydrolyzed per gram (dry weight) of root tissue in the time indicated. A longer time was necessary with turnip tissue to obtain sufficient hydrolysis for accurate determination.

No correlation was found between myrosin activity and resistance to clubroot.

TABLE 4.—*Myrosin activity of black mustard and turnip root tissue*

| Determination No. | Variety or collection | Resistance | Milligrams of sinigrin hydrolyzed per gram of root tissue (dry weight) in— | | |
|---|---|---|---|---|---|
| | | | 30 minutes | 120 minutes | 480 minutes |
| 1 [1] | Black mustard No. 3 | Resistant | 17.4 | 26.4 | |
| | Black mustard No. 10 | do | 11.6 | 23.7 | |
| | Black mustard No. 8 | Susceptible | 11.0 | 24.2 | |
| | Black mustard No. 14 | do | 13.4 | 17.3 | |
| 2 [2] | Black mustard No. 3 | Resistant | 3.0 | 8.3 | 29.1 |
| | Black mustard No. 14 | Susceptible | 3.3 | 5.5 | 18.1 |
| | | | 165 | 165 | |
| | | | Outer tissue | Inner tissue | |
| 3 [3] | Turnip T–113 | Resistant | 11.6 | 5.5 | |
| | Turnip 1–115–1x2B | Susceptible | 23.6 | 5.0 | |

[1] Plants grown in greenhouse, entire root system used for analysis.
[2] Plants grown under field conditions, mature roots analyzed.
[3] Turnip roots were divided into outer tissue (largely periderm and phloem), and inner tissue (xylem), and each portion was analyzed separately. Time of hydrolysis 165 minutes in all cases.

## DISCUSSION

This investigation was undertaken because it had been claimed that specific volatile mustard oils in certain crucifers were responsible for their resistance to the clubroot organism. The mustard oils which occur in the roots of turnip, cabbage, horseradish, black mustard, and white mustard were isolated and characterized. This study showed that the more commonly known mustard oil, allyl isothiocyanate, which occurs in the seeds of black mustard in the form of the glycoside sinigrin, is not found in the roots of any of these crucifers except horseradish. On the other hand, another mustard oil, β-phenethyl isothiocyanate, was found in the root tissue of each of the crucifers mentioned. Presumably this oil occurs also as a glycoside and is

formed by hydrolysis of the glycoside by the proper enzyme system when the tissue is macerated. It has the same general degree of toxicity toward fungi as allyl isothiocyanate.

An intensive study was made of resistant and susceptible strains of black mustard and turnip. Quantitative estimation of the total amount of mustard oils in the roots of these plants failed to show correlation of isothiocyanate content to resistance or susceptibility to clubroot. This fact might be interpreted as showing either that the mustard oils, though toxic to the pathogen, were not responsible for resistance; or that the enzyme system in the susceptible plants did not release the free toxic oils from the nontoxic glycosides at the proper time or rate to be effective in imparting resistance to the tissue. A further study of this thioglucoside-splitting enzyme system in resistant and susceptible root systems showed no tangible relationship of enzyme concentration or activity to resistance or susceptibility.

It is thus evident that, although mustard oils that are highly toxic to fungus organisms, including the clubroot organism, may be obtained from the roots of many crucifers, they do not seem to inhibit infection or development of the pathogen in these tissues. Furthermore, they are not responsible in any demonstrable way for the resistance of certain varieties and strains to the clubroot organisms. An opinion is thereby reemphasized which has been previously expressed (*32*), viz, that the presence of a toxic substance in the tissue is not proof that it functions in the phenomenon of disease resistance.

## SUMMARY

This investigation deals with the role of mustard oils in resistance to clubroot infection in the crucifers. A biochemical study has been made of the isothiocyanate content of root tissue as related to species and varietal resistance to invasion by *Plasmodiophora brassicae* Wor. β-phenethyl isothiocyanate has been isolated from root tissue of resistant and susceptible strains of turnip (*Brassica rapa*) and black mustard (*B. nigra*) and from susceptible strains of white mustard (*B. alba*) and horseradish (*Armoracia rusticana*). β-phenethyl isothiocyanate appears to be the principal mustard oil in the root tissue of many crucifers, both those susceptible and those resistant to clubroot.

Previous methods for estimating mustard oil were found to give variable recoveries when applied to the estimation of the isothiocyanate produced by the enzymatic hydrolysis of a mustard-oil glycoside. Improved analytical methods for estimating isothiocyanates were developed and applied to a study of (1) the enzymatic hydrolysis of sinigrin, (2) the isothiocyanate content of turnip and mustard root tissue as it relates to resistance to clubroot, and (3) the thioglucosidase (myrosin) activity of resistant and susceptible root tissues. Quantitative estimation of the total mostard-oil content and the relative myrosin activity of root tissues failed to show any correlation of isothiocyanate content or thioglucosidase activity to resistance or susceptibility to clubroot.

From these studies it appears that the mustard oils are not essential in enabling certain varieties of crucifers to prevent or retard clubroot development in their root tissues.

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# EFFECT OF CHANGE OF TEMPERATURE ON RELATIVE TOXICITY OF ROTENONE AND PHENOL[1]

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## INTRODUCTION

In toxicological studies conducted by the United States Department of Agriculture in which the goldfish (*Carassius auratus*) has been used as the test animal, the temperature regularly employed has been 27° C., as specified when this method of study was described (*6*).[3] This temperature may have to be lowered, however, because of the rapid development of bacterial diseases in fish stock kept at such a high temperature in the dechlorinated water that is now used. Consequently, experiments were undertaken in the hope of finding some orderly relationships that might permit correlation of comparisons at one temperature with those at another.

## EXPERIMENTAL PROCEDURE

The test substances chosen were rotenone and phenol, because these had been used as standards of comparison in previous work and also because they differ greatly in type of toxic action, the former being toxic to goldfish at very low concentrations but relatively slow in action, and the latter requiring much higher concentrations and being much more rapid in action. The samples used were chemically pure. All test solutions of phenol were made from a 5-percent aqueous stock solution, but those of rotenone were made as usual from acetonic stock solutions. The highest concentration of rotenone showed a faint opalescence, indicating that the limit of solubility had been passed.

Toxicological tests were made on each compound at a constant temperature in the usual manner (*6*)—that is, survival time was determined at a number of concentrations—but in addition a third variable was introduced by repeating the tests at different temperature levels. The goldfish, a single lot obtained from one pond and weighing between 2 and 4 gm. each, were acclimated to laboratory conditions, having been held at 7° C. for 6 weeks, and were active and feeding well despite the low temperature. Tests were run at a constant temperature of 7°, and then the temperature of the stock

[1] Received for publication July 1, 1942.
[2] The writer is now with the Division of Control Investigations.
[3] Italic numbers in parentheses refer to Literature Cited, p. 80.

tanks was raised slowly to 12° and held there for a week before the tests at this level were begun. The procedure was then repeated, the temperature being raised in 5° increments up to 27°, and the fish were acclimatized by holding them at each temperature a week before starting the tests at that level. This acclimatization has an important stabilizing effect on the survival time.

Since the elapsed time before testing, and therefore the age of the test animal, increases as the temperature is raised, any changes may be thought to be a function of age as well as temperature. The writer has found, however, that for a relatively long-lived organism, such as the goldfish, held under the conditions of these tests, such a variation in age causes no significant difference in toxicity measurements.

## EXPERIMENTAL RESULTS

### TOXICITY IN RELATION TO TEMPERATURE

The results of the tests are assembled in tables 1 and 2. A ready graphical comparison of the toxic action as expressed by these data is permitted by the survival-time and velocity-of-fatality curves in figures 1 and 2. The reciprocal nature of the two types of curves resulting from the use of the geometric mean allowed the use of intermediate points, obtained by interpolation, where they were of advantage in locating parts of the velocity-of-fatality curves.

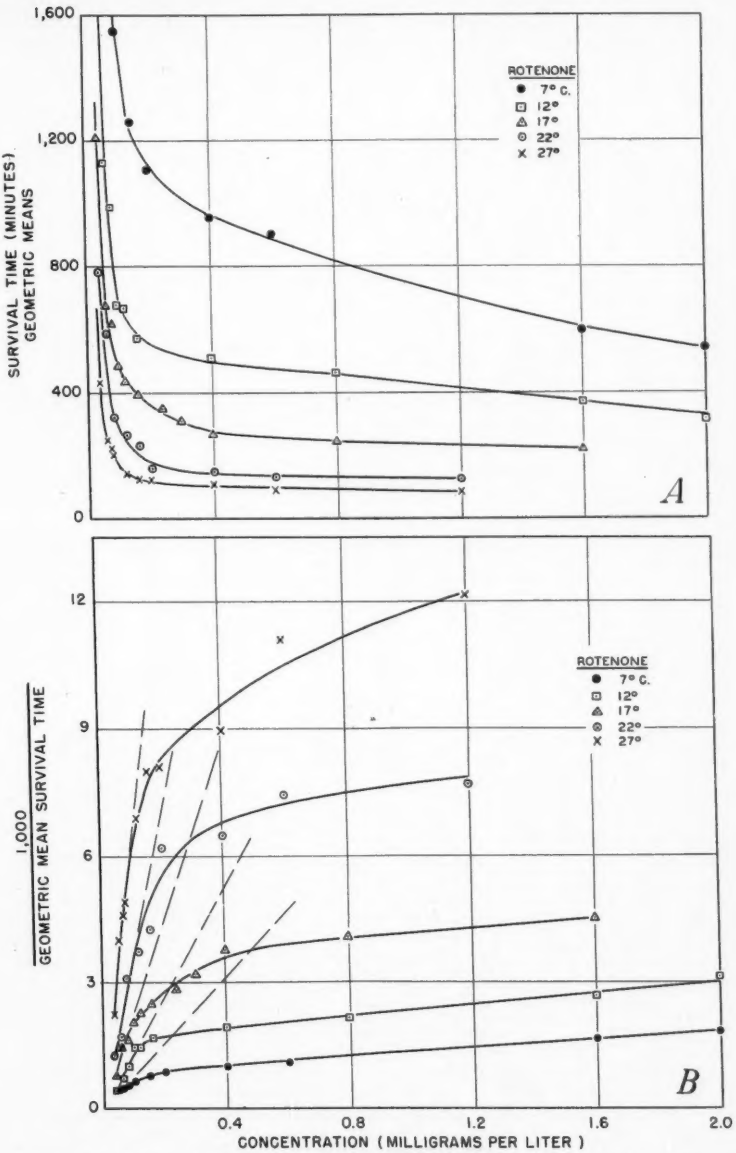


FIGURE 1.—Survival-time (*A*) and velocity-of-fatality (*B*) curves for rotenone.

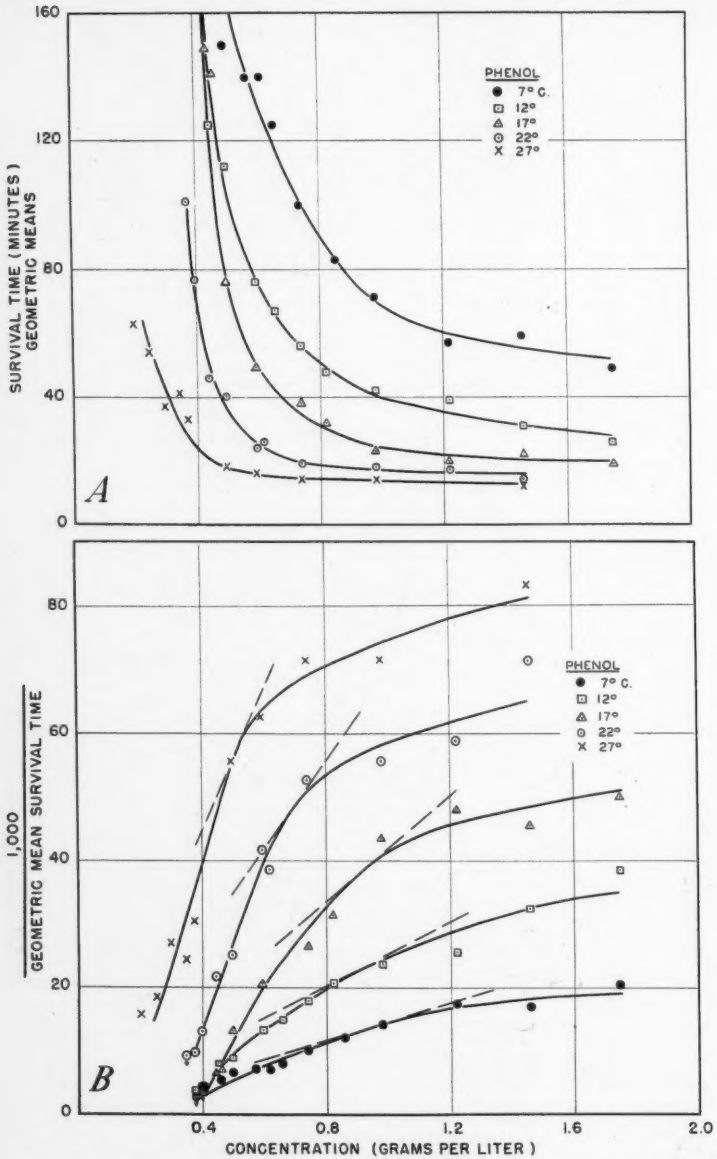


FIGURE 2.—Survival-time (*A*) and velocity-of-fatality (*B*) curves for phenol.

TABLE 1.—*Toxic action of rotenone on goldfish at different temperatures*

| Temperature (°C.) | Concentration | Fishes used | Mean length of fishes | Mean [1] weight of fishes | Mean survival time [2] | | 1,000÷ geometric mean | Mean of 1,000÷ survival time |
|---|---|---|---|---|---|---|---|---|
| | | | | | Arithmetic | Geometric | | |
| | *Milligrams per liter* | *Number* | *Millimeters* | *Grams* | *Minutes* | *Minutes* | | |
| 7.0±0.4 | 3.20 | 10 | 41 | 2.1 | 564±20 | 557 ×÷ 1.04 | 1.80 | 1.81 |
| | 2.00 | 14 | 42 | 2.3 | 541±12 | 537 ×÷ 1.02 | 1.86 | 1.87 |
| | 1.60 | 13 | 43 | 2.4 | 604±15 | 600 ×÷ 1.02 | 1.67 | 1.68 |
| | .600 | 13 | 43 | 2.4 | 922±17 | 918 ×÷ 1.02 | 1.09 | 1.10 |
| | .400 | 13 | 43 | 2.4 | 968±22 | 963 ×÷ 1.02 | 1.04 | 1.05 |
| | .200 | 15 | 43 | 2.4 | 1,117±16 | 1,115 ×÷ 1.01 | .897 | .900 |
| | .150 | 16 | 44 | 2.6 | 1,278±41 | 1,262 ×÷ 1.03 | .792 | .801 |
| | .100 | 12 | 45 | 2.8 | 1,614±84 | 1,552 ×÷ 1.05 | .644 | .663 |
| | .0800 | 12 | 44 | 2.6 | 1,883±116 | 1,801 ×÷ 1.06 | .555 | .574 |
| | .0600 | 11 | 43 | 2.4 | 2,244±183 | 2,117 ×÷ 1.07 | .472 | .494 |
| | .0500 | 12 | 45 | 2.8 | 2,340±174 | 2,210 ×÷ 1.07 | .452 | .474 |
| | .0200 | 12 | 45 | 2.8 | [3] | | | |
| 12.0±0.4 | 2.00 | 12 | 43 | 2.4 | 330±17 | 318 ×÷ 1.05 | 3.14 | 3.24 |
| | 1.60 | 12 | 44 | 2.6 | 385±23 | 368 ×÷ 1.07 | 2.72 | 2.85 |
| | .800 | 15 | 43 | 2.4 | 488±30 | 462 ×÷ 1.07 | 2.16 | 2.30 |
| | .400 | 16 | 44 | 2.6 | 533±28 | 510 ×÷ 1.05 | 1.96 | 2.04 |
| | .160 | 16 | 41 | 2.1 | 586±23 | 576 ×÷ 1.04 | 1.74 | 1.79 |
| | .120 | 30 | 43 | 2.4 | 691±22 | 669 ×÷ 1.03 | 1.49 | 1.54 |
| | .096 | 16 | 45 | 2.8 | 709±41 | 675 ×÷ 1.05 | 1.48 | 1.53 |
| | .0800 | 16 | 43 | 2.4 | 1,051±72 | 991 ×÷ 1.07 | 1.009 | 1.059 |
| | .0600 | 16 | 42 | 2.3 | 1,191±80 | 1,131 ×÷ 1.06 | .884 | .901 |
| | .0400 | 14 | 42 | 2.3 | 1,842±101 | 1,767 ×÷ 1.06 | .566 | .587 |
| | .0100 | 16 | [4] | [4] | [5] | | | |
| 17.0±0.3 | 1.60 | 16 | 43 | 2.4 | 223±7 | 220 ×÷ 1.03 | 4.55 | 4.61 |
| | .800 | 16 | 42 | 2.3 | 248±8 | 244 ×÷ 1.03 | 4.10 | 4.18 |
| | .400 | 15 | 43 | 2.4 | 275±13 | 264 ×÷ 1.04 | 3.79 | 3.94 |
| | .300 | 17 | 43 | 2.4 | 319±15 | 309 ×÷ 1.05 | 3.24 | 3.34 |
| | .240 | 17 | 43 | 2.4 | 365±18 | 351 ×÷ 1.05 | 2.85 | 2.96 |
| | .160 | 17 | 42 | 2.3 | 414±22 | 398 ×÷ 1.05 | 2.51 | 2.62 |
| | .120 | 20 | 42 | 2.3 | 461±25 | 437 ×÷ 1.06 | 2.29 | 2.40 |
| | .100 | 20 | 43 | 2.4 | 512±29 | 485 ×÷ 1.05 | 2.06 | 2.16 |
| | .0800 | 17 | 43 | 2.4 | 656±42 | 618 ×÷ 1.06 | 1.62 | 1.70 |
| | .0600 | 18 | 42 | 2.3 | 725±46 | 678 ×÷ 1.06 | 1.47 | 1.55 |
| | .0400 | 16 | 45 | 2.8 | 1,360±134 | 1,209 ×÷ 1.05 | .827 | .923 |
| | .0100 | 16 | [4] | [4] | [6] | | | |
| 22.0±0.3 | 1.20 | 15 | 43 | 2.4 | 128±5 | 126 ×÷ 1.04 | 7.94 | 8.15 |
| | .600 | 15 | 44 | 2.6 | 143±11 | 133 ×÷ 1.06 | 7.52 | 7.95 |
| | .400 | 14 | 43 | 2.4 | 153±5 | 150 ×÷ 1.04 | 6.67 | 6.76 |
| | .200 | 13 | 43 | 2.4 | 168±10 | 161 ×÷ 1.06 | 6.21 | 6.45 |
| | .160 | 17 | 44 | 2.6 | 245±13 | 235 ×÷ 1.05 | 4.26 | 4.44 |
| | .120 | 17 | 43 | 2.4 | 284±16 | 268 ×÷ 1.06 | 3.73 | 3.99 |
| | .0800 | 18 | 43 | 2.4 | 344±19 | 323 ×÷ 1.06 | 3.10 | 3.33 |
| | .0600 | 12 | 43 | 2.4 | 619±38 | 596 ×÷ 1.07 | 1.68 | 1.76 |
| | .0353 | 17 | 45 | 2.8 | 855±60 | 784 ×÷ 1.07 | 1.28 | 1.38 |
| | .0100 | 12 | 44 | 2.6 | [7] | | | |
| 27.0±0.2 | 1.20 | 14 | 43 | 2.4 | 83±2 | 82 ×÷ 1.03 | 12.2 | 12.4 |
| | .600 | 15 | 43 | 2.4 | 90±3 | 90 ×÷ 1.03 | 11.1 | 11.3 |
| | .400 | 13 | 43 | 2.4 | 112±4 | 111 ×÷ 1.03 | 9.01 | 9.13 |
| | .200 | 18 | 41 | 2.1 | 128±6 | 123 ×÷ 1.04 | 8.13 | 8.33 |
| | .160 | 16 | 40 | 2.0 | 130±7 | 125 ×÷ 1.05 | 8.00 | 8.25 |
| | .120 | 15 | 41 | 2.1 | 148±4 | 145 ×÷ 1.03 | 6.90 | 7.02 |
| | .0800 | 16 | 43 | 2.4 | 223±19 | 203 ×÷ 1.09 | 4.93 | 5.42 |
| | .0750 | 18 | 43 | 2.4 | 240±16 | 224 ×÷ 1.07 | 4.46 | 4.75 |
| | .0600 | 15 | 42 | 2.3 | 262±15 | 249 ×÷ 1.06 | 4.02 | 4.22 |
| | .0400 | 14 | 45 | 2.8 | 506±51 | 439 ×÷ 1.13 | 2.28 | 2.73 |
| | .0100 | 12 | [4] | [4] | [8] | | | |

[1] Estimated from length, which measurement excludes the caudal fin.
[2] The limits of error indicated are probable errors of the means. In the case of the arithmetic means they are differences from the means; in the case of the geometric means they are ratios to the means.
[3] 3 killed in 40 hours, 2 slightly affected; 7 apparently unaffected after 50 hours.
[4] The fishes were not measured but were of approximately the same size.
[5] 1 killed in 30½ hours; others apparently unaffected after 51 hours.
[6] Apparently unaffected in 51 hours.
[7] Apparently unaffected after 50 hours.
[8] Apparently unaffected after 36 hours.

TABLE 2.—*Toxic action of phenol on goldfish at different temperatures*

| Temperature (° C.) | Concentration | Fishes used | Mean length of fishes | Mean [1] weight of fishes | Mean survival time [2] | | 1,000÷ geometric mean | Mean of 1,000÷ survival time |
|---|---|---|---|---|---|---|---|---|
| | | | | | Arithmetic | Geometric | | |
| | *Grams per liter* | *Number* | *Millimeters* | *Grams* | *Minutes* | *Minutes* | | |
| 7.0±0.3 | 1.75 | 10 | 43 | 2.4 | 50±2 | 49 ×/÷ 1.04 | 20.4 | 20.7 |
| | 1.46 | 10 | 42 | 2.3 | 61±3 | 59 ×/÷ 1.06 | 16.9 | 17.4 |
| | 1.22 | 10 | 42 | 2.3 | 59±3 | 57 ×/÷ 1.05 | 17.5 | 18.0 |
| | .980 | 12 | 40 | 2.0 | 73±3 | 71 ×/÷ 1.04 | 14.1 | 14.4 |
| | .855 | 12 | 44 | 2.6 | 85±5 | 83 ×/÷ 1.06 | 12.0 | 12.5 |
| | .739 | 10 | 42 | 2.3 | 103±7 | 100 ×/÷ 1.07 | 10.0 | 10.4 |
| | .655 | 14 | 43 | 2.4 | 129±7 | 125 ×/÷ 1.06 | 8.00 | 8.33 |
| | .617 | 12 | 42 | 2.3 | 143±4 | 140 ×/÷ 1.03 | 7.14 | 7.14 |
| | .570 | 13 | 43 | 2.4 | 143±5 | 141 ×/÷ 1.04 | 7.09 | 7.24 |
| | .495 | 10 | 43 | 2.4 | 156±10 | 150 ×/÷ 1.07 | 6.67 | 6.91 |
| | .454 | 12 | 44 | 2.6 | 186±6 | 184 ×/÷ 1.03 | 5.43 | 5.51 |
| | .397 | 14 | 42 | 2.3 | 223±11 | 217 ×/÷ 1.05 | 4.61 | 5.17 |
| | .372 | 11 | [3] | [3] | [4] >360 | >360 | <2.78 | <2.78 |
| 12.0±0.3 | 1.75 | 10 | 44 | 2.6 | 27±1 | 26 ×/÷ 1.04 | 38.5 | 38.8 |
| | 1.46 | 10 | 41 | 2.1 | 32±2 | 31 ×/÷ 1.06 | 32.3 | 33.1 |
| | 1.22 | 11 | 43 | 2.4 | 40±2 | 39 ×/÷ 1.04 | 25.6 | 26.2 |
| | .980 | 10 | 44 | 2.6 | 43±2 | 42 ×/÷ 1.05 | 23.8 | 24.6 |
| | .820 | 11 | 45 | 2.8 | 50±3 | 48 ×/÷ 1.05 | 20.8 | 21.4 |
| | .739 | 13 | 41 | 2.1 | 59±3 | 56 ×/÷ 1.06 | 17.9 | 18.7 |
| | .658 | 12 | 43 | 2.4 | 69±4 | 67 ×/÷ 1.07 | 14.9 | 15.7 |
| | .593 | 10 | 42 | 2.3 | 79±4 | 76 ×/÷ 1.06 | 13.2 | 13.7 |
| | .495 | 12 | 42 | 2.3 | 114±5 | 112 ×/÷ 1.04 | 8.93 | 9.24 |
| | .446 | 15 | 44 | 2.6 | 136±10 | 125 ×/÷ 1.09 | 8.00 | 8.93 |
| | .397 | 12 | 44 | 2.6 | 248±17 | 233 ×/÷ 1.08 | 4.29 | 4.57 |
| | .372 | 14 | 45 | 2.8 | [5] 283±24 | 260 ×/÷ 1.09 | 3.85 | 4.13 |
| 17.0±0.2 | 1.75 | 10 | 43 | 2.4 | 20±1 | 20 ×/÷ 1.05 | 50.0 | 51.8 |
| | 1.46 | 11 | 44 | 2.6 | 22±1 | 22 ×/÷ 1.06 | 45.5 | 48.0 |
| | 1.22 | 11 | 43 | 2.4 | 22±1 | 21 ×/÷ 1.04 | 47.6 | 46.6 |
| | .980 | 10 | 42 | 2.3 | 23±2 | 23 ×/÷ 1.06 | 43.5 | 44.4 |
| | .820 | 12 | 42 | 2.3 | 33±2 | 32 ×/÷ 1.06 | 31.3 | 32.7 |
| | .739 | 14 | 43 | 2.4 | 39±2 | 38 ×/÷ 1.06 | 26.3 | 27.8 |
| | .593 | 11 | 42 | 2.3 | 51±2 | 49 ×/÷ 1.05 | 20.4 | 20.8 |
| | .495 | 10 | 42 | 2.3 | 84±7 | 76 ×/÷ 1.10 | 13.2 | 15.1 |
| | .461 | 14 | 40 | 2.0 | 181±26 | 141 ×/÷ 1.14 | 7.09 | 8.56 |
| | .441 | 15 | 43 | 2.4 | 167±14 | 149 ×/÷ 1.09 | 6.71 | 7.58 |
| | .397 | 27 | [3] | [3] | [6] >325 | >290 | <3.44 | 4.0– 3.3 |
| | .372 | 14 | [3] | [3] | [7] >360 | >300 | <3.33 | 3.4– 1.9 |
| 22.0±0.2 | 1.46 | 10 | 42 | 2.3 | 14±1 | 14 ×/÷ 1.05 | 71.4 | 74.2 |
| | 1.22 | 10 | 44 | 2.6 | 18±1 | 17 ×/÷ 1.04 | 58.8 | 58.0 |
| | .980 | 10 | 43 | 2.4 | 18±1 | 18 ×/÷ 1.06 | 55.6 | 58.4 |
| | .739 | 13 | 43 | 2.4 | 20±1 | 19 ×/÷ 1.06 | 52.6 | 54.1 |
| | .617 | 6 | 44 | 2.6 | 26±1 | 26 ×/÷ 1.03 | 38.5 | 39.1 |
| | .593 | 12 | 40 | 2.0 | 25±2 | 24 ×/÷ 1.06 | 41.7 | 43.6 |
| | .495 | 19 | 43 | 2.4 | 44±3 | 40 ×/÷ 1.09 | 25.0 | 27.7 |
| | .441 | 13 | 42 | 2.3 | 50±4 | 46 ×/÷ 1.10 | 21.7 | 23.8 |
| | .397 | 15 | 45 | 2.8 | 82±5 | 77 ×/÷ 1.06 | 13.0 | 13.5 |
| | .372 | 24 | 43 | 2.4 | 122±12 | 101 ×/÷ 1.10 | 9.90 | 11.8 |
| | .346 | 15 | 42 | 2.3 | [8] >204 | >110 | <9.09 | 11.1–10.5 |
| 27.0±0.2 | 1.46 | 10 | 41 | 2.1 | 12±1 | 12 ×/÷ 1.04 | 83.3 | 86.7 |
| | .980 | 10 | 43 | 2.4 | 14±1 | 14 ×/÷ 1.04 | 71.4 | 75.0 |
| | .739 | 12 | 41 | 2.1 | 14±1 | 14 ×/÷ 1.04 | 71.4 | 74.5 |
| | .593 | 13 | 41 | 2.1 | 17±1 | 16 ×/÷ 1.07 | 62.5 | 67.7 |
| | .495 | 14 | 40 | 2.0 | 20±2 | 18 ×/÷ 1.09 | 55.6 | 59.8 |
| | .372 | 14 | 42 | 2.3 | 35±3 | 33 ×/÷ 1.06 | 30.3 | 32.2 |
| | .346 | 16 | 39 | 1.9 | 43±2 | 41 ×/÷ 1.07 | 24.4 | 27.3 |
| | .298 | 16 | 42 | 2.3 | 60±11 | 37 ×/÷ 1.11 | 27.0 | 32.3 |
| | .249 | 16 | 39 | 1.9 | 66±6 | 54 ×/÷ 1.08 | 18.5 | 22.0 |
| | .199 | 14 | 40 | 2.0 | 85±14 | 63 ×/÷ 1.11 | 15.9 | 18.6 |
| | .025 | 6 | [3] | [3] | [9] | | | |

[1] Estimated from length, which measurement excludes the caudal fin.
[2] The limits of error indicated are probable errors of the means. In the case of the arithmetic means they are differences from the means; in the case of the geometric means they are ratios to the means.
[3] The fishes were not measured but were of approximately the same size.
[4] 27 percent killed in 8 hours.
[5] 86 percent killed in 8 hours.
[6] 63 percent killed in 8 hours.
[7] 50 percent killed in 8 hours.
[8] 67 percent killed in 8 hours.
[9] Slight effect but not lethal in 8 hours.

Since this work was designed to study toxicity well within the range of toxic concentrations, little information was obtained which bears on the difficultly determined thresholds of toxicity. It is obvious that within the range studied there is a decided increase in apparent toxicity with increase in temperature between 7° and 27° C.

### QUANTITATIVE COMPARISON OF TOXICITY

For the comparisons of toxicity the measure proposed by the writer in 1935 (7) and used in subsequent work was adopted. This measure, the minimal product of concentration and survival time, ignores the tolerance parameters and considers toxicity only in the range of most efficient action with respect to both variables. Its value may be determined by calculation from the survival-time curve or graphically from the velocity-of-fatality curve by drawing the maximal tangent to the curve from the origin, as indicated with broken lines. In the latter case the value of the slope of this line is the reciprocal of the minimal product, and expresses the greatest ratio of dilution to survival time.

The data for comparison by this criterion are given in table 3. The minimal product of concentration and survival time is designated by $c_m t_m$ and, since toxicity varies inversely with this value, its reciprocal is also given. Its coordinates, $c_m$ and $t_m$, are given so that the region approximating this condition may be located readily in the graphs. The ratio of increase of toxicity is based on the toxicity of phenol at 7° C. as unity. The value for $1/c_m t_m$ used as the base for the rotenone series was 8.12 liters per gram per minute, calculated with the aid of the mean value for relative toxicity, 0.00181. This procedure was used because the determination of the actual value for rotenone at 7° must have a relatively large error, owing to the difficulty of reading such long survival times and the use of the comparatively small number of fishes.

TABLE 3.—*Relative toxicity of rotenone and phenol at different temperatures*

| Temperature (°C.) | $c_m$ | $t_m$ | $c_m t_m$ | Toxicity $\frac{1}{c_m t_m}$ | Relative toxicity phenol to rotenone | Ratio of increase of toxicity | Relative effect of 5° increment |
|---|---|---|---|---|---|---|---|
| | *Milligrams per liter* | *Minutes* | *Gram-minutes per liter* | *Liters per gram per minute* | *Percent* | | |
| **ROTENONE** | | | | | | | |
| 7 | 0.045 | 2,500 | 0.113 | 8.85 | | 1.09 | |
| 12 | .080 | 910 | .0728 | 13.7 | | 1.69 | 1.55 |
| 17 | .070 | 600 | .0420 | 23.8 | | 2.93 | 1.73 |
| 22 | .080 | 350 | .0280 | 35.7 | | 4.40 | 1.50 |
| 27 | .070 | 223 | .0156 | 64.1 | | 7.89 | 1.80 |
| **PHENOL** | | | | | | | |
| 7 | 1,000 | 68 | 68.0 | 0.0147 | 0.166 | 1.00 | |
| 12 | 900 | 44 | 39.6 | .0253 | .185 | 1.72 | 1.72 |
| 17 | 960 | 25 | 24.0 | .0417 | .175 | 2.84 | 1.65 |
| 22 | 660 | 21 | 13.9 | .0719 | .201 | 4.89 | 1.72 |
| 27 | 520 | 17 | 8.84 | .113 | .176 | 7.69 | 1.57 |
| Mean of all ratios | | | | | .181 | | 1.66 |

## STATISTICAL ANALYSIS OF CORRELATION BETWEEN RELATIVE TOXICITY AND TEMPERATURE

Because of the nice relationships found in the foregoing study of the freehand curves, it is desirable to go beyond the mere determination of an average of variation of the mean survival times and seek information on the precision of the minimal *ct* measurements.

If the minimal *ct* measurements are estimates of a parameter defining a characteristic of this population, there should be some orderly relationship between the relative toxicities of the two compounds at different temperatures. If such a relationship is sought between two substances of such different mode of action, it seems logical to exclude from the statistical comparison the extremes of action, where the two variables vary so disproportionately. To the extreme left in the direction of diminishing concentrations, the usefulness of time measurements decreases until at the thresholds the comparison can be one of concentration alone and the ratio of relative toxicity is expanded its widest in favor of the compound evincing greater toxicity from the concentration aspect—in this case rotenone. To the extreme right in the direction of increasing concentrations, the usefulness of concentration measurements decreases until the comparison may become one of time alone, and if one of the compounds is a relatively fast-acting toxicant, as is phenol in this case, not only may the ratio vary greatly but even the order of toxicity may be reversed. To bring into consideration the spheres of such adverse influences will at least weaken the stability of the parameter to be estimated by this criterion. Therefore, in each case the concentrations selected for statistical treatment from the data in tables 1 and 2 are in a range which corresponds to as many points on the curve as affect the location and evaluation of the minimal *ct* point without bringing in the adverse effects of the extremes. This range was judged to be delimited by the use of 140 percent of the $c_m t_m$ values (table 3) as the maximal product of concentration and survival time.

To evaluate the sampling errors of the $1/c_m t_m$ estimations, curves that might fit the variates were studied. A semilog curve, obtained with the use of logarithms of concentrations and reciprocals of survival times, was found to be well adapted to such treatment. Straight lines were fitted by the method of least squares to the data within the stated range of concentrations. These regression lines are drawn and the complete semilog curves indicated in figure 3. The regression equations are given below. For rotenone $X$ is 1,000 times the concentration in milligrams per liter; for phenol $X$ is 100 times the concentration in grams per liter. For both compounds $Y$ is 1,000 divided by the survival time in minutes.

| Temperature (° C.) | For rotenone | For phenol |
|---|---|---|
| 7 | $Y = -0.7646 + 0.7152 \log X$ | $Y = -41.04 + 27.56 \log X$ |
| 12 | $Y = -2.748 + 2.068 \log X$ | $Y = -75.14 + 50.02 \log X$ |
| 17 | $Y = -3.623 + 2.864 \log X$ | $Y = -109.2 + 74.13 \log X$ |
| 22 | $Y = -8.241 + 5.994 \log X$ | $Y = -151.4 + 107.5 \log X$ |
| 27 | $Y = -10.964 + 8.539 \log X$ | $Y = -206.3 + 153.1 \log X$ |

The minimal products are given by those tangents to the curves expressed by these equations which pass through the origin; their values are determined with the use of the knowledge that these tangents are the first differential coefficients of the curves.

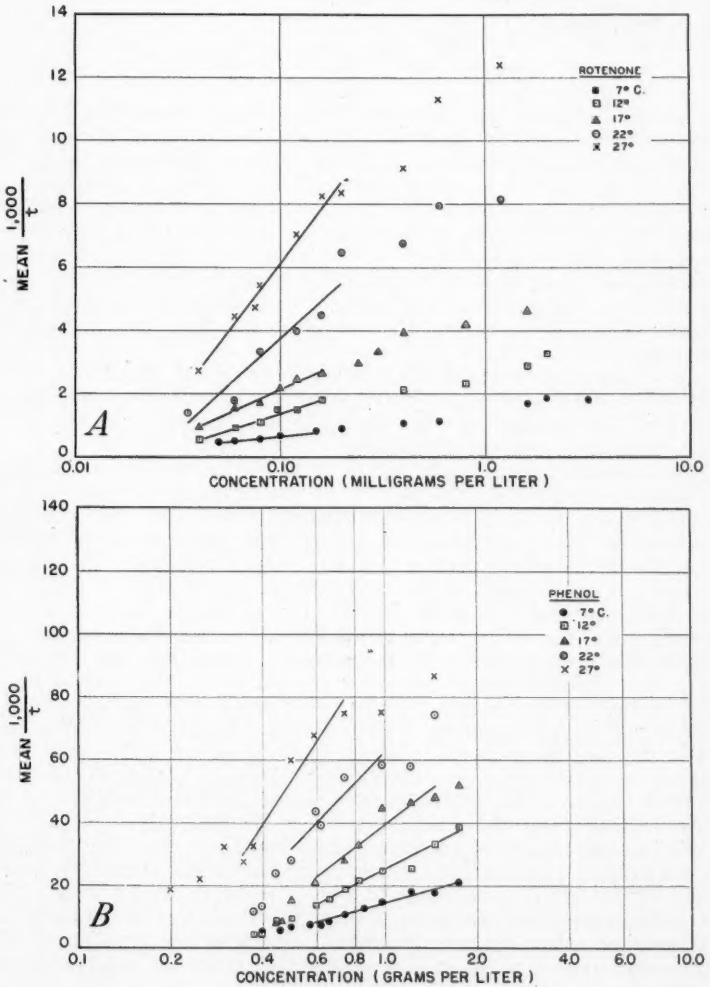


FIGURE 3.—Regression of reciprocal of time on logarithm of concentration for rotenone (*A*) and phenol (*B*) at different temperatures.

Since the curves are completely specified by two factors, slope and position, the error estimates of each factor will contribute to the error of the minimal *ct* products. The latter may be calculated in two ways [4]: (1) From the method of extremes, using a sampling range of $\pm 1$ standard error for each factor and combining them; or (2) from the equation

$$S \text{ (in percent)} = \frac{230.3\sqrt{S_{\bar{y}}^2 + (\bar{x} - \log c_m)^2 . S_b^2}}{b}$$

in which $S$ denotes the standard error of the reciprocal of $c_m t_m$; $S_{\bar{y}}$, the standard error of the mean reciprocal of survival time; $\bar{x}$, the mean log concentration; $c_m$, the concentration corresponding to the minimal *ct*; $S_b$, the standard error of the regression coefficient, $b$. $S$ is actually the relative error of $1/t_m$, but since all the error is derived from the determination of the value of this variate, it may be regarded as the relative error of the reciprocal of $c_m t_m$. The first method also permits ready estimation of the sampling variation of location of the minimal *ct* product. The two methods gave practically identical results, and the second was, in fact, used throughout as a check.

The results of this statistical analysis are given in table 4. The ratios for increase of toxicity were calculated in the same manner as for table 3. In this case, however, the base for the rotenone series is 9.038 liters per gram per minute.

It is apparent that, insofar as the actual ratios are concerned, there is little choice between the method of freehand curves based on geometric mean survival times and concentration and the statistical method of linear regression computed from reciprocals of survival time and logarithm of concentration. There is a definite and regular increase, though small, in the $1/c_m t_m$ values for rotenone obtained by the latter method over those obtained by the former, because in such distributions the reciprocal of the harmonic mean is always slightly larger than the reciprocal of the geometric mean, and the difference near the critical point is relatively greater in the case of rotenone. This difference results in a slightly lower mean (0.16 percent) for the toxicity of phenol as compared with that of rotenone, than is obtained with the use of geometric means (0.18 percent). The statistical method produces ratios showing slightly more regular effects of increase in temperature. The errors for the minimal *ct* products estimated by this method show the phenol values to be determined with more precision than those for rotenone; those for the former vary from 2.5 to 4.2 percent whereas those for the latter vary from 5.0 to 7.4 percent from 12° to 27° C. and jump to 14.3 percent at the 7° level. The determination in the last instance was difficult because of the necessity for reading extremely long survival times, and the location of the $c_m t_m$ point appears to be, in a sense, slightly extrapolative as recorded.

[4] The equation was developed by C. M. Smith and the other method was suggested by F. M. Wadley, both of this Bureau. Grateful acknowledgment is also made for other helpful suggestions by them in the statistical treatment of the data.

TABLE 4.—*Statistical analysis of the effect of temperature on the relative toxicity of rotenone and phenol when measured by the minimal ct product*

TABLE 4.—*Statistical analysis of the effect of temperature on the relative toxicity of rotenone and phenol when measured by the minimal ct product*

ROTENONE

| Temperature (°C.) | $c_m$ | $t_m$ | $c_m t_m$ | Toxicity $\frac{1}{c_m t_m}$ | Relative toxicity phenol to rotenone | Ratio of increase of toxicity $R_T$ | Relative effect of 5° increment $Q_5$ | Relative effect of 10° increment $Q_{10}$ |
|---|---|---|---|---|---|---|---|---|
| | *Milligrams per liter* | *Minutes* | *Gram-minutes per liter* | *Liters per gram per minute* | *Percent* | | | |
| 7 | 0.03187±0.00861 | 3,219 | 0.1026±0.01471 | 9.747±1.46 | | 1.08 | | |
| 12 | .05798± .00713 | 1,114 | .06459± .00325 | 15.48± .79 | | 1.71 | 1.59±0.24 | |
| 17 | .05003± .00840 | 804 | .04022± .00297 | 24.86±1.86 | | 2.75 | 1.61± .14 | 2.55±0.41 |
| 22 | .06443± .00884 | 384 | .02475± .00159 | 40.40±2.61 | | 4.47 | 1.63± .16 | 2.61± .21 |
| 27 | .05226± .00764 | 270 | .01409± .00097 | 70.97±4.95 | | 7.85 | 1.76± .17 | 2.85± .29 |
| PHENOL | | | | | | | | |
| 7 | 838.3± 57.7 | 83.5 | 70.03±1.74 | 0.01428±0.00036 | 0.147±0.021 | 1.00 | | |
| 12 | 864.1± 79.4 | 46.0 | 39.77±1.18 | .02514± .00075 | .162± .009 | 1.76 | 1.76±0.07 | |
| 17 | 807.6±105.0 | 31.1 | 25.08± .92 | .03987± .00147 | .160± .013 | 2.79 | 1.59± .07 | 2.79±0.12 |
| 22 | 696.2±109.3 | 21.4 | 14.91± .61 | .06707± .00273 | .166± .013 | 4.70 | 1.68± .09 | 2.67± .13 |
| 27 | 605.0± 56.8 | 15.0 | 9.10± .38 | .1099 ± .00458 | .155± .013 | 7.70 | 1.64± .09 | 2.76± .15 |
| Mean of all ratios | | | | | .158±0.0064 | | 1.66±0.05 | 2.71±0.10 |

## DISCUSSION

Two conclusions can be drawn at once from the results. Despite the great dissimilarity in the toxic action of rotenone and phenol, when measured by this criterion the increase in toxicity with increase in temperature is proportionately the same for each compound, and the ratio of increase is the same for each 5° increment between 7° and 27° C. It follows that rotenone and phenol have the same relative toxicity when measured at any temperature level in this range. The geometric increase in relative toxicity with arithmetic increase in temperature is shown in figure 4. The equation for both compounds, determined by the method of least squares, is

$$R_T = 0.515e^{0.100T}$$

in which $R_T$ is the ratio of toxicity at temperature $T°$ to that at 7°. When the values are expressed in liters per gram per minute, the specific equations for toxicity at temperature $T°$ are, for rotenone,

$$\left(\frac{1}{c_m t_m}\right)_T = 4.66e^{0.100T}$$

and for phenol,

$$\left(\frac{1}{c_m t_m}\right)_T = 0.00736e^{0.100\ T}.$$

Thus, for each compound, the logarithm of the value for toxicity is a linear function of the temperature.

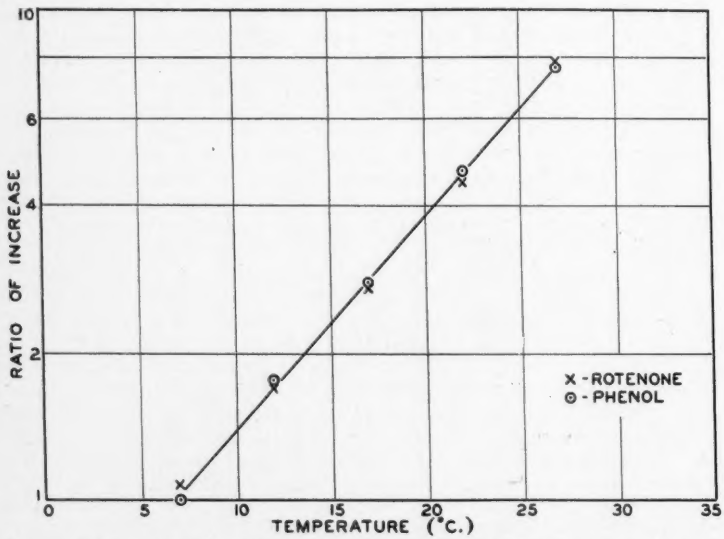


FIGURE 4.—Increase in toxicity of rotenone and phenol with increase in temperature in the range 7° to 27° C.

If relative toxicity as measured by this method is independent of the temperature at which comparisons are made, the temperature effects found above must be due solely to acceleration of one or more physiological processes in the test animal. In this case the con-

trolling factor would most likely be a respiratory phenomenon. To make comparisons with temperature coefficients for respiratory processes reported in the literature (*2, 4, 5, 9, 10, 11*), the ratio of increase of effect for a 10° C. increase in temperature ($Q_{10}$) was calculated for the two substances, as shown in table 4.

It may be noted that the increase in toxicity is 2.7-fold for a rise of 10°, and is so nearly constant that we most likely have to do here with a simple and not a complex reaction. This value is about the usual ratio of increase in the velocity of a chemical reaction with rise in temperature. In fact, the results obtained over the temperature range given agree well with the relation between the rate of a chemical reaction and temperature as expressed by Van't Hoff's equation (*8*)

$$V_1 = V_0 e^{c(t_1 - t_0)}$$

where $V_1$ and $V_0$ are the velocities of the reaction at temperatures $t_1$ and $t_0$, respectively. The improved modification of this equation making use of absolute temperature and the gas constant, the Arrhenius equation (*1*),

$$V_1 = V_0 e^{\frac{\mu}{2}\left(\frac{t1 - t0}{t0t1}\right)}$$

is reported to express more accurately the effect of temperature on many biological reactions (*3, p. 270*). This equation does express the data nearly as well for phenol with a range of values for the thermal characteristic, $\mu$ (determined for the four 5° increments), of 15,100 to 17,900 and a mean of 17,000 calories per mole. Although for rotenone there is a slight increase in $Q_5$ with increase in temperature (table 4) which is reflected in the slight concavity upwards of a curve through the rotenone points in figure 7, these differences are obviously not significant. However, since the trend is in the reverse direction to the condition for which the Arrhenius modification is a correction, the range of $\mu$ values for rotenone when this equation is applied becomes relatively greater, 14,700 to 19,800, with a mean of 16,600 calories per mole. Calculated from the equation of the curve in figure 4, the range of values for $\mu$ for the same 5° increments is from 15,800 to 17,600, with a mean of 16,900 calories per mole.

In this connection it may be mentioned that Ege and Krogh (*5*), studying the effect of temperature change on oxygen consumption (measured in cubic centimeters per kilogram per minute) of a single 9.3-gm. goldfish, reported results that were practically the same whether or not the fish was narcotized with urethane. $Q_{10}$ was found to be approximately 2.7 in the temperature range 5° to 28° C. If calculated according to the Arrhenius equation, $\mu$ is found to be about 16,000 calories per mole. However, Ege and Krogh concluded that, since there was a steady and considerable decrease in $Q_{10}$ from 0° to 28° C., Van't Hoff's temperature coefficient did not express the relationship satisfactorily.

Wells (*11*) came to the same conclusion after studying the relationship between temperature and oxygen uptake but with the Pacific killifish (*Fundulus parvipinnis*) as the test animal.

Sumner and Doudoroff (*10*), using the goby (*Gillichthys mirabilis*) and taking the rate of death from each of two lethal agents, potassium cyanide of a single concentration (0.001 molar in sea water) and boiled sea water, as a measure of respiratory metabolism, reported that the

reciprocal of the survival time varied logarithmically with the temperature in the range 10° to 30° C., $Q_{10}$ lying between 2.6 and 2.8 with the first agent and, with much less constancy, between 2.3 and 3.0 with the second.

Crozier and Stier (*4*) reported that the temperature characteristic for the frequency of rhythmic opercular movements in six quiescent goldfish, averaging 60 mm. in length (probably over-all), was 16,500 calories.

Bělehrádek (*2*), after a thorough survey of the work prior to 1930 on the use of temperature coefficients in connection with biological phenomena, considered the application of chemical temperature-velocity formulas to have failed.

Study of the application of these two formulas to the data obtained by the writer has emphasized a fundamental consideration that is usually ignored. $Q_{10}$ is constant for both compounds when toxicity is measured by the minimal product, $c_m t_m$, and is practically constant when velocity is measured by $1/t$ at any one concentration near $c_m$. However, $Q_{10}$ gradually tends to increase with increase in temperature if velocities are measured at concentrations below $c_m$ but, on the other hand, to decrease with increase in temperature if measured at higher concentrations. The results of work by Powers (*9*) on the effect of temperature change on the toxicity of lithium chloride to goldfish (weight 1 to 2 gm.), though of an approximate nature, agree with the first half of this statement. Whether measured by survival time or by the slope of the theoretical velocity-of-fatality curve (the straight line approximating that part of the velocity-of-fatality curve near its point of inflection), or whether calculated from a temperature-relative toxicity curve, increase of $Q_{10}$ with rise in temperature is indicated. The range of concentrations studied is at the left of the concentration that would correspond to a minimal *ct* product; that is, the product (within experimental error) increases with decrease in concentration. Moreover, comparisons by the theoretical velocity-of-fatality curve will always be comparisons at concentrations to the left of that of the minimal *ct* product.

When velocities are compared at some concentration other than that of the minimal *ct* product, this type of instability for $Q_{10}$ is a natural condition for such sigmoid curves as complete velocity-concentration curves if they converge as they approach the limiting tolerance factors. The values for the thermal increment, $\mu$, would vary with the concentration, although in this study the variation is slight. Therefore, just as the comparison of isotherms describing biological reactions in the dimensions time and concentration required study of the effect of change of temperature, so the comparisons of reactions in the dimensions time and temperature require study of the effect of change in concentration. Thermal increments are usually reported without explanation as to why that particular concentration was used; $\mu$ may or may not be the same at some other concentration.

It appears, then, that of the physiological reactions affected by change in temperature the main or controlling effect is on the respiratory processes of the test animal. In consequence, variation of apparent toxicity with change in temperature seems to be due solely to a response of the test animal; relative toxicity as inherent in the nature of the compound, when measured by this method, is the same regardless of the temperature level at which measured, provided, of course,

that the limits of the range are not so extreme as to bring in other influences. In other words, relative toxicity, as shown by rotenone and phenol with this method, is independent of the temperature level at which measured.

## CONCLUSIONS

(1) The use of the minimal product of concentration and survival time as a criterion of relative toxicity measures a parameter of the test population with respect to these two variables with such definiteness that introduction of another variable, such as temperature, does not affect the value obtained for relative toxicity with that particular population. This does not mean that all goldfish of the same size and age will give the same value as found above, for there is evidence from previous work by the author that fish from different ponds and of different ancestry give a different value. But there is also evidence that fish of uniform size and age from any one pond and of the same ancestry, even though taken from populations in different years, give values not greatly different. The value of relative toxicity thus has a stability that has not been shown to be obtainable with other measures.

(2) Under the conditions of these tests relative toxicity may be defined as a function of the nature of chemical compounds and its values are not affected by change of temperature. Such a change influences only the physiological processes of the test animal, and the apparent effect on toxic action is a function of the animal's resistance. In this sense toxicity and resistance do not have a simple inverse relationship but are distinct conceptions, the former inherent in the nature of the toxicant and the latter inherent in the nature of the test animal.

## SUMMARY

The effect of temperature on apparent toxicity, when measured by the criterion of the minimal product of concentration and survival time, has been studied, with rotenone and phenol as the test materials and small goldfish as the test animals. The experiments were conducted at temperatures ranging from 7° to 27° C. in 5° increments. All results were subjected to statistical analysis.

Despite the great dissimilarity in the mode of toxic action of rotenone and phenol, increase in toxicity with increase in temperature, when measured by this criterion, was found to be proportionately the same for the two compounds. Phenol was 0.16 percent as toxic as rotenone at any temperature in this range.

The ratio of increase was found to be the same for equal increments in temperature, 1.66 for a 5° increment.

The geometric increase of relative toxicity ($R_T$) with arithmetic increase in temperature ($T$° C.) is summarized by the equation

$$R_T = 0.515e^{0.100T}.$$

When expressed in liters per gram per minute, the value of toxicity at $T$° C. for rotenone is $4.66e^{0.100T}$, and for phenol is $0.00736e^{0.100T}$.

Toxicity and resistance do not have a simple inverse relationship but are distinct conceptions, the former inherent in the nature of the toxic compound and the latter inherent in the nature of the test animal.

Resistance only is affected by change in temperature. Relative toxicity is a function of the chemical compound and remains the same at any level within a range of temperatures not deleterious to the test animal.

The criterion of relative toxicity, the minimal product of concentration and survival time, therefore measures a definite characteristic of the test population.

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